UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

ANA PAULA CRESTANI

INVESTIGANDO OS APRENDIZADOS SUBSEQUENTES:
MECANISMOS PLÁSTICOS E DEPENDÊNCIA TEMPORAL

Porto Alegre/RS
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Tese apresentada ao Programa de Pós-graduação em Neurociências da Universidade Federal do Rio Grande do Sul como requisito final para obtenção do título de doutora.

Área de concentração: Neurociências

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DEDICATÓRIA

As pessoas que sempre me apoiaram, que são meus maiores e melhores exemplos, meus pais.

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EPÍGRAFE

There are many hypotheses in science which are wrong.
That's perfectly all right: it's the aperture to finding out what's right.
Science is a self-correcting process.

Carl Sagan

RESUMO

A formação de memórias de medo contextuais, como as estudadas no presente trabalho, requer a indução da plasticidade sináptica iniciada pela ativação de receptores transmembrana localizados nos neurônios de estruturas encefálicas como o hipocampo. O fluxo iônico mediado pelos receptores N-metil-D-aspartato (NMDARs) é essencial para ativar vias de sinalização intracelular que darão suporte à formação da memória. No entanto, esses receptores parecem não ser necessários em situações onde os animais passaram por uma experiência prévia similar a que está sendo aprendida. Dessa forma, um aprendizado anterior pode modificar os mecanismos de plasticidade que serão utilizados para codificar uma nova informação, caracterizando um fenômeno de metaplasticidade. Esse fenômeno ocorre quando os animais são pré-expostos ao local onde posteriormente serão submetidos a um aprendizado associativo ou quando são re-submetidos a mesma tarefa comportamental com dicas contextuais/espaciais diferentes. No presente trabalho, investigamos (i) os mecanismos de plasticidade sináptica (receptores) e de plasticidade não-sináptica (excitabilidade neuronal) recrutados para a formação do segundo aprendizado e (ii) se a independência dos NMDARs é mantida quando a memória anterior foi adquirida remotamente.

Os animais utilizados nesse trabalho (camundongos ou ratos) foram expostos a dois aprendizados sequenciais realizados na tarefa de condicionamento aversivo ao contexto (CAC). O intervalo entre os condicionamentos foi de dois dias nos experimentos do Capítulo I e de três ou quarenta dias nos experimentos do Capítulo II. Cada aprendizado ocorreu em uma caixa de condicionamento com características próprias de formato, odor e iluminação (contexto A ou contexto B), sendo que o primeiro aprendizado ocorreu no contexto A e o segundo no contexto B.

Nos experimentos do Capítulo I foram avaliadas no hipocampo dorsal as modificações na excitabilidade neuronal hipocampal induzidas pelo primeiro condicionamento, bem como os receptores envolvidos com a aquisição da memória subsequente e a sobreposição neuronal entre os dois aprendizados. Com a utilização do camundongo transgênico Teg-Tag foi possível identificar os neurônios recrutados para o primeiro aprendizado. Esse animal tem a expressão da proteína fluorescente verde (GFP, do inglês, *green fluorescent protein*) controlada pela ativação do gene *c-fos*, que é fisiologicamente transcrito após a atividade neuronal. Dessa forma, os neurônios ativados pelo aprendizado são marcados com GFP. Através da técnica de *patch clamp* foi observado que os neurônios GFP+ mantiveram a excitabilidade elevada por até dois dias após o treinamento no CAC. Além disso, a identificação dos neurônios recrutados

para o aprendizado subsequente foi realizada através da marcação imunofluorescente da proteína Fos, no seu pico de expressão endógena, noventa minutos após o re-treino. Foi observada uma maior sobreposição neuronal (GFP+, Fos+) quando os animais foram re-treinados no mesmo contexto dois dias após o primeiro treino. Uma sobreposição intermediária (GFP+, Fos+) foi vista quando os animais tiveram o segundo condicionamento no contexto B, sendo ela significativamente maior do que a sobreposição nos animais não re-treinados. Adicionalmente, foi demonstrado que a aquisição do aprendizado subsequente é mediada por receptores metabotrópicos glutamatérgicos (mGluRs) ao invés de NMDARs.

No Capítulo II foi investigado se uma memória remota, adquirida há quarenta dias, ainda seria capaz de influenciar nos mecanismos de plasticidade recrutados para aquisição do aprendizado subsequente. A dinâmica da consolidação sistêmica foi considerada nesses experimentos já que a evocação da memória remota passa a depender de estruturas encefálicas neocorticais, sem recrutar a atividade hipocampal. Apesar da evocação da memória remota não requerer a atividade hipocampal, foi observado que a aquisição do aprendizado subsequente a uma memória remota necessita a atividade de pelo menos uma sub-região do hipocampo (dorsal ou ventral). Complementarmente, os resultados indicaram que, quando o intervalo entre os aprendizados é aumentado (de três para quarenta dias), a formação do aprendizado subsequente, que era independente de NMDARs, volta a depender da plasticidade sináptica mediada por esses receptores no hipocampo (dorsal e ventral).

Juntos, nossos resultados sugerem que o primeiro aprendizado causa um aumento da excitabilidade neuronal e modifica a plasticidade sináptica recrutada para o aprendizado subsequente, sendo este último mediado por mGluRs ao invés de NMDARs. Além disso, a metaplasticidade induzida pelo primeiro condicionamento é transiente; quando o intervalo entre as exposições é aumentado, o segundo aprendizado passa a depender novamente da ativação dos NMDARs.

PALAVRAS-CHAVE: aprendizados subsequentes, NMDAR, mGluR, hipocampo, córtex cingulado anterior, metaplasticidade

ABSTRACT

Contextual fear memory formation, like the ones explored in the current work, requires the induction of the synaptic plasticity mediated by the activation of transmembrane receptors that are present in the brain structures as the hippocampus. The ionic flux through the N-methyl-aspartate-D-aspartate is crucial for activation of the intracellular signaling pathways that will support memory formation. However, these receptors are not necessary when animals had a prior similar learning. In this way, a previous learning can modify the plasticity mechanism that will be recruited to encode a new information, featuring a metaplasticity phenomenon. This phenomenon occurs when animals are pre-exposed to an environment where they will learn an associative learning later or when animals are re-exposed to the same behavioral task with distinct contextual/spatial cues. In the present study, we investigated (i) the synaptic plasticity mechanisms (receptors) and the non-synaptic plasticity mechanisms (neuronal excitability) required for the acquisition of the second learning and (ii) whether a subsequent learning that occurs in a remote time-point is still NMDAR-independent.

The animals used in this study (mice or rats) were exposed to two sequential learnings that were performed in the contextual fear conditioning (CFC). The interval between conditionings were two days in the experiments of Chapter I and three or forty days in the experiments of the Chapter II. Each learning was performed in a box with differences on shape, odor and illumination (context A or context B). The first learning occurred in the context A followed by learning on context B.

In the experiments of Chapter I it was evaluated the changes in the hippocampal neuronal excitability induced by the first conditioning, the receptors involved with the acquisition of the subsequent memory and the neuronal overlapping between the two sequential learnings. The Teg-Tag transgenic mouse allowed to identify the neurons activated for the first learning experience. This animal has the GFP expression under control of c-fos promoter that is activated by neuronal activity. It was shown by patch clamp that GFP+ neurons are still more excitable two days after learning. Also, the identification of neurons recruited for the subsequent learning was made through immunofluorescent staining of the Fos protein in its peak of endogenous expression, ninety minutes after learning. A greater overlapping (GFP+, Fos+) was observed when animals were retrained in the same context two days after first training. An intermediate overlapping was observed when animals were conditioned in the context B and this expression was significantly higher when compared to animals that were not

retrained in either context. Additionally, it was shown that acquisition of the subsequent learning is mediated by metabotropic glutamate receptors (mGluRs) instead of NMDARs

In the Chapter II it was investigated whether a remote memory, acquired forty days earlier, is still able to influence in the synaptic plasticity mechanisms recruited for the acquisition of the subsequent learning. Systems consolidation dynamics was considered in these experiments because memory retrieval of a remote memory depends on neocortical brain regions, it not requires hippocampal activity. It was confirmed that hippocampus is not necessary for remote memory retrieval, however at least one longitudinal division of the hippocampus (dorsal or ventral) is essential for learning following a prior remote memory. Moreover, the results indicate that acquisition of the second learning is once again mediated by NMDARs in the hippocampus when the interval between learnings is extended from three to forty days.

Altogether, our results suggest that the first learning lead to an increase in the neuronal excitability and modify the synaptic plasticity mechanism recruited for following learning, mGluR are required instead of NMDAR. Furthermore, the metaplasticity induced by first conditioning is transient; the second learning once again requires NMDARs activation when the interval between learnings is longer.

KEY-WORDS: subsequent learning, NMDAR, mGluR, hippocampus, anterior cingulate cortex, metaplasticity

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

AMPAR	a-amino-3-hydroxy- 5-methyl-4-isoxazolepropionic acid receptor
AP5	(2R)-amino-5-phosphonopentanoate
BLA	basolateral amygdala
CA	do grego, Cornu Ammonis
cAMP	cyclic adenosine monophosphate
CaM	calmodulin
CaMK	Ca2+/CaM-dependent kinases
CE	central amygdala
CFC	contextual fear conditioning
CI-AMPAR	calcium impermeable AMPAR
CP-AMPAR	calcium permeable AMPAR
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
CS	conditioned stimulus
DAG	diacylglycerol
DG	dentate gyrus
ERK	extracellular-signal-regulated-kinase
Gi	proteína G inibitória
Gq	proteínas G estimulatória
HFS	high frequency stimulation
IEG	immediate early genes
iGluRs	ionotropic glutamate receptors
IP3	inositol 1,4,5-triphosphate
KAR	kainate receptor
LFS	low frequency stimulation
LTD	long-term depression
LTP	long-term potentiation
mGluRs	metabotropic glutamate receptors
MTT	multiples traces theory
NMDAR	N-methyl-D-aspartate receptor
PKC	protein kinase C
PLC	fosfolipase C
SCT	standard consolidation theory
STDP	spike-timing dependent plasticity
TGRA	temporally-graded retrograde amnesia
US	unconditioned stimulus

SUMÁRIO

1. INTRODUÇÃO

1.1. Memória

1.1.1. Definição e classificação

Num sentido amplo, a memória pode ser definida como a capacidade de armazenar e acessar futuramente as informações. Semon foi o cientista que definiu a existência desses dois aspectos básicos da memória, ainda no início do século XX. Apesar de já se discutir naquela época a existência do 'traço da memória', a maioria dos estudiosos focavam sobre o processo de aprendizado e armazenamento, mas não sobre o lembrar (evocar). Nesse sentido, a teoria de Semon foi inovadora. Ele postulava a existência do engrama e da ecforia (do inglês, *ecphory*). Enquanto o engrama compreenderia o substrato fisiológico onde a informação é armazenada, a ecforia seria relativa ao processo de evocação, onde o engrama sairia do seu estado latente. Dessa forma, a memória emergiria da interação entre o que foi armazenado (engrama) e as dicas que levariam ao processo de evocação (ecforia). Apesar desses termos terem sido cunhados por Semon, a popularização do termo engrama foi impulsionada pelos trabalhos de Lashley. Em busca de tentar compreender onde a memória estava armazenada, Lashley concluiu que ela encontrava-se difusa por todo o encéfalo, já que o aumento da extensão das lesões encefálicas em roedores causava prejuízos proporcionalmente maiores, independentemente de onde as lesões fossem realizadas (Josselyn *et al*, 2015a).

Nessa mesma época, os neurocirurgiões Penfield e Rasmussen obtiveram as primeiras evidências de que memórias poderiam estar localizadas em regiões específicas. Eles observaram, em procedimentos pré-cirúrgicos realizados para determinar a região geradora das crises epilépticas, que estimulações elétricas aplicadas no lobo temporal eram suficientes para induzir a recordação de memórias vívidas. Um estudo de caso especialmente importante para determinar a necessidade do lobo temporal na formação e evocação das memórias, foi o do paciente Henry Molaison (H.M.). Ele desenvolveu epilepsia refratária a medicamentos após um acidente na infância e suas crises intensificaram-se ao longo dos anos, levando os médicos (Scoville e Milner) a optarem pela ressecção bilateral da região geradora das crises, que incluiu o hipocampo e áreas adjacentes do lobo temporal medial. Depois da cirurgia, o paciente apresentou melhora do quadro clínico, mas como efeito colateral foi observado um grande prejuízo na formação de novas memórias (amnésia anterógrada), além de amnésia retrógrada para memórias recentes, mantendo-se apenas as memórias de procedimentos.

A observação de que a ressecção de diferentes estruturas encefálicas prejudicava memórias de diferentes modalidades, foi uma das responsáveis pelo desenvolvimento de uma classificação para os diferentes tipos de memória. Na classificação, são levadas em consideração principalmente (i) a natureza das informações que as memórias codificam e (ii) o tempo que elas perduram.

Quanto à natureza das informações, as memórias são usualmente classificadas em (i) explícitas ou declarativas e (ii) implícitas ou procedurais. As memórias explícitas são facilmente formadas, mas também facilmente esquecidas; são conscientemente relembradas e, em humanos, declaradas verbalmente. Já as memórias implícitas requerem uma formação gradual, mas dificilmente são esquecidas; elas são consideradas difíceis de declarar, já que são relacionadas a habilidades motoras ou hábitos. Memórias da categoria explícita são dependentes de estruturas como o hipocampo e o neocórtex. Já memórias implícitas requerem a atividade de estruturas como o cerebelo e os núcleos da base (Kandel *et al*, 2014). Importante ressaltar que, apesar da classificação facilitar o estudo e o entendimento, uma memória pode possuir tanto componentes explícitos como implícitos, recrutando a atividade conjunta das estruturas encefálicas subjacentes a cada tipo de memória.

Quanto ao tempo, algumas memórias adquiridas podem ser momentâneas, enquanto outras podem perdurar décadas (Dudai and Morris, 2013; Kandel *et al*, 2014; Katche *et al*, 2013). Um dos principais fatores modulatórios da durabilidade da memória é o caráter emocional atribuído a situação que o animal está vivenciando. Dependendo da relevância inferida, a probabilidade desse evento ser armazenado no sistema nervoso vai variar, podendo ou não estabelecer um traço de memória e, se estabelecido, o traço pode ser efêmero ou duradouro (Ploski and McIntyre, 2015). A memória de trabalho é o tipo menos durável, persistindo apenas de segundos a minutos. A memória de curta duração dura de minutos a horas. Já as memórias de longa duração chegam a persistir por várias horas, dias ou anos. Genericamente, a memória de trabalho pode ser sustentada por estruturas como o córtex pré-frontal, já memórias de curta e de longa duração podem ser mantidas pelo hipocampo (Kandel *et al*, 2000).

1.1.2. Formação da memória

A formação da memória costuma ser dividida em duas fases. A aquisição, em que as informações consideradas relevantes são codificadas pelo sistema nervoso central, e a

consolidação, onde o traço da memória passa por um processo de estabilização, ficando resistente a interferências. Os mecanismos moleculares subjacentes às diferentes fases da memória são distintos e muitos deles foram estudados através de manipulações farmacológicas em variados momentos entre o treino (aprendizado) e o teste (evocação). Diz-se que um fármaco interfere na aquisição quando ele é administrado antes do aprendizado, afetando ambas as memórias de curta e longa duração. Quando o fármaco é administrado antes ou após o treino e não tem efeito na memória de curta duração, interferindo apenas na de longa, é dito que ele está agindo sob a consolidação da memória (Johansen *et al*, 2011).

1.1.3. Consolidação da memória

O termo consolidação foi cunhado por Muller and Pilzecker no final do século XVIII. A consolidação costuma ser dividida em sináptica e sistêmica. A primeira estabiliza as conexões dos circuitos locais (Dudai, 2004; Kandel and Pittenger, 1999) ao passo que a última é um processo de reorganização gradual das regiões encefálicas que sustentam a memória (Dash *et al*, 2004; Debiec *et al*, 2002; Frankland and Bontempi, 2005; Nadel and Moscovitch, 1997).

Acredita-se que a janela da consolidação sináptica dure cerca de 6 horas (Izquierdo *et al*, 2006; De Oliveira Alvares *et al*, 2008), podendo ser modificada por fatores como aversividade (Casagrande et al., 2018). Após o término da consolidação sináptica, a memória deixa de ser suscetível a interferências e é dita consolidada. Uma série de mecanismos moleculares e celulares, como ativação de segundos mensageiros, expressão gênica, síntese proteica e remodelamento sináptico são desencadeados pela consolidação sináptica (Johansen *et al*, 2011). Muitos desses mecanismos são dependentes da ativação de receptores NMDA (do inglês, *N-methyl-D-aspartate receptor*, NMDAR) e são semelhantes aos observados durante o fenômeno de potenciação de longa duração (LTP, do inglês, *long-term potentiation*) (Kandel *et al*, 2014), que será melhor explicado adiante.

Os NMDARs são considerados detectores de coincidência porque são ativados quando existe atividade pré-sináptica juntamente com despolarização pós-sináptica. A abertura deles permite a entrada de cálcio, este atua como segundo mensageiro de diversas vias de sinalização intracelular importantes para a plasticidade sináptica e para a formação da memória (Malenka and Nicoll, 1999). O aumento do cálcio intracelular leva a fosforilação da CaMKII (do inglês, *Ca^{2+}/calmodulin-dependent protein kinase II*) (Kandel, 2012), que pode alterar o tráfego de

receptores AMPA (do inglês, *a-amino-3-hydroxy- 5-methyl-4-isoxazolepropionic acid*, AMPAR) nos espinhos dendríticos, além de induzir a fosforilação de subunidades específicas desse receptor, levando ao aumento a força sináptica (Malinow and Malenka, 2002). O aumento do Ca^{2+} e/ou a ativação da CaMKII induz a atividade de cinases como a PKA (do inglês, *protein kinase A*), PKC (do inglês, *protein kinase C*) e MAPK (do inglês, mitogen-activated protein kinases). A MAPK pode ser ativada direta ou indiretamente pela PKA, PKC e CaMKII (Kandel, 2012; Kandel *et al*, 2000). Outra cinase que parece ser muito importante para a persistência da memória é a PKMξ (do inglês, *protein kinase M zeta*), uma isoforma da PKC. Ela é responsável por manter as subunidades GluA2 do AMPAR na sinapse. Essas subunidades influenciam a permeabilidade do AMPAR, quando elas estão presentes o receptor é impermeável ao Ca^{2+}, sendo esse o possível motivo pelo qual as memórias ficam mais estáveis e persistem por mais tempo (Sacktor, 2008). Diversos estudos têm demonstrado que a síntese de RNA e de proteínas também são muito importantes para a formação da memória de longa duração (Davis and Squire, 1984; Hernandez and Abel, 2008; McGaugh, 2000). Igualmente importantes são os fatores de transcrição, como o CREB (do inglês, *cAMP responsive element binding protein*). O CREB pode ser fosforilado por todas as cinases supracitas, promovendo a transcrição de genes dependentes de CRE (do inglês, *cAMP responsive element*) que estão relacionados à plasticidade (Alberini, 2009) **(Figura 1)**.

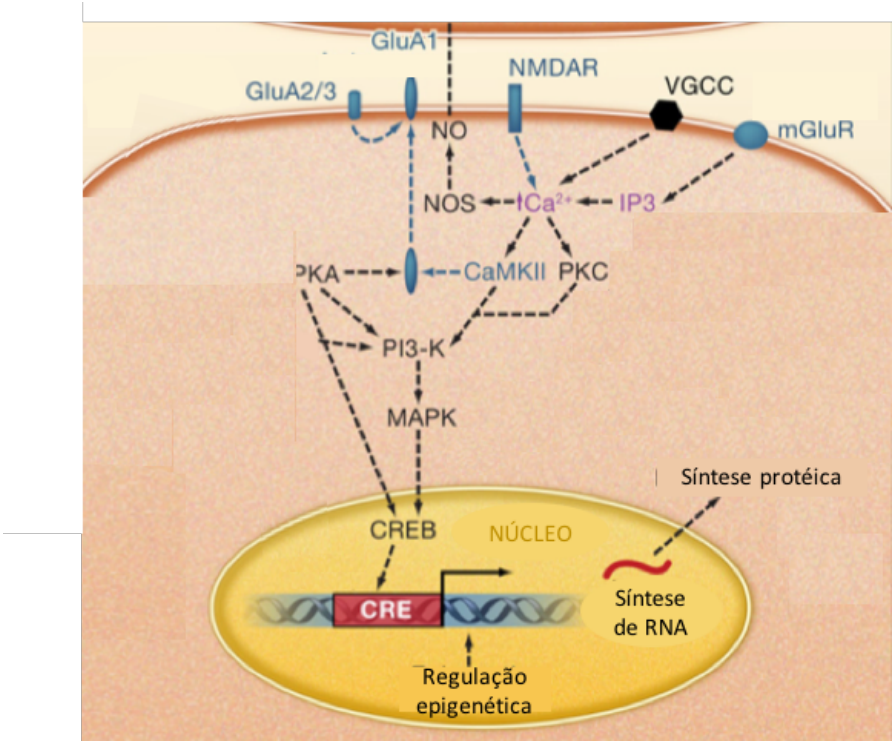

Figura 1 – Vias de sinalização envolvidas com a formação da memória. Modificada de Johansen et al., 2011.

Genes de expressão imediata

Em resposta à atividade sináptica e, consequente, atividade neuronal, alguns genes são rapidamente ativados, os chamados genes de expressão imediata (do inglês, *immediate early genes*, IEG). A expressão deles tem um pico em torno de trinta a sessenta minutos após o aprendizado e é reduzida aproximadamente aos cento e vinte minutos (Cullinan et al, 1995). Esses genes podem ser induzidos por cAMP (do inglês, *cycli adenosine monophosphate*) e Ca^{2+}, por exemplo. O mediador chave no controle da expressão dos IEGs é o CREB, ele medeia a ativação transcricional do sítio CRE que está presente na região promotora de quase todos os genes de expressão imediata, incluindo *c-fos*, BDNF, egr-1 (ou zif268), homer1a/vesl1s e Arc (Okuno, 2011). Tanto o mRNA quanto a proteína, essa última com pico de expressão entre sessenta e noventa minutos após o aprendizado (Zangenehpour and Chaudhuri, 2002), são comumente usados como correlatos de atividade neuronal recente (Guzowski *et al*, 2001). No presente trabalho, a marcação imunofluorescente da proteína Fos foi utilizada para quantificar os neurônios recentemente ativados **(Figura 2)**.

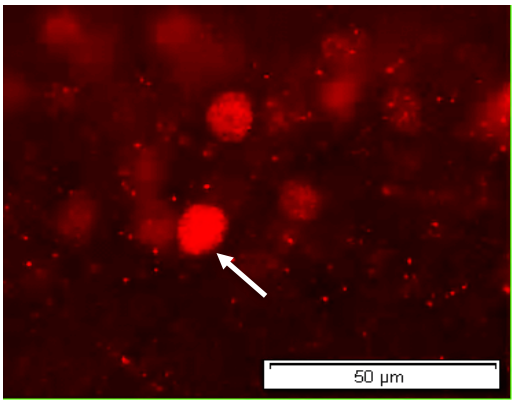

Figura 2 – Imagem representativa da marcação imunofluorescente da proteína Fos no corpo celular de neurônios da camada piramidal de CA1. A seta indica um corpo celular com a marcação bem evidente.

Além das modificações moleculares, a formação da memória também pode causar modificações estruturais, como alterações na densidade e morfologia dos espinhos dendríticos (Kitanishi *et al*, 2009; Matsuzaki *et al*, 2004; Sanders *et al*, 2012), que não serão aprofundadas aqui.

1.1.4. Consolidação sistêmica

Algumas teorias têm sido propostas para tentar explicar a reorganização das regiões encefálicas que sustentam a memória. A teoria padrão da consolidação sistêmica (do inglês,

standard consolidation theory, STC) foi a primeira a surgir. Ela é embasada principalmente na observação de pacientes que após sofrerem algum dano cerebral apresentavam amnésia retrógrada para eventos recentes (do inglês, *temporally-graded retrograde amnesia*, TGRA) (Kritchevsky and Squire, 1989). A SCT prediz que as memórias se tornam independentes do hipocampo ao longo do tempo (Squire and Alvarez, 1995; Squire and Zola, 1998). Durante o aprendizado aconteceriam modificações sinápticas rápidas no hipocampo e modificações lentas no neocórtex. A reativação dos circuitos hipocampais fortaleceria as conexões hipocampo-corticais e, com o passar do tempo, as conexões cortico-corticais passariam a sustentar a memória (McClelland *et al*, 1995; Squire and Alvarez, 1995). Essa teoria também obteve bastante sustentação em estudos com modelos animais, onde lesões ou inativação hipocampal causavam amnésia retrógrada apenas para eventos recentes (Barry *et al*, 2016; Broadbent and Clark, 2013; Kim and Fanselow, 1992; Sierra *et al*, 2017; Tayler and Wiltgen, 2013). Dessa forma, segundo a SCT, o hipocampo agiria como um local de armazenamento temporário da memória, sendo as regiões neocorticais o repositório final **(Figura 3)**.

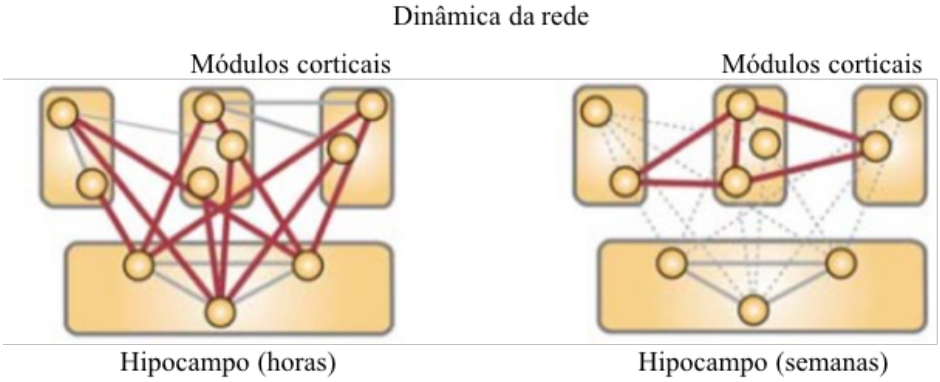

Figura 3 – Dinâmica da consolidação sistêmica da memória. Adaptada de Frankland & Bontempi, 2005.

Uma ideia diferente foi proposta por Nadel and Moscovitch (1997), chamada de teoria dos múltiplos traços (do inglês, *multiples traces theory*, MTT). A MTT diferencia as memórias em episódicas (detalhadas) ou semânticas (generalizadas), postulando que as memórias episódicas são sempre dependentes do hipocampo. Então, o grau de envolvimento hipocampal não seria determinado pela idade da memória, mas sim pelo tipo da memória que está sendo avaliado (semântica ou episódica) e pela extensão da lesão hipocampal. A ideia da extensão é embasada em estudos com pacientes que apresentam amnésia retrógrada, onde lesões maiores ocasionam déficits mais pronunciados de memória. Do ponto de vista dessa teoria, lesões hipocampais incompletas afetam preferencialmente memórias recentes porque elas são mais

fracas (menos estáveis), já lesões completas levam a eliminação de ambas as memórias recentes e remotas (Nadel and Moscovitch, 1997).

Recentemente, baseado na natureza qualitativa da memória, Winocur propôs uma nova teoria, a da transformação da memória (Winocur *et al*, 2007). Ela sugere que a modificação das estruturas que suportam a memória ao longo do tempo é acompanhada por modificações correspondentes na qualidade da memória. Dessa forma, a independência hipocampal faria com que uma memória detalhada se tornasse generalizada e dependente de estruturas corticais (Winocur and Moscovitch, 2011).

A discussão entre essas teorias ainda é atual e todas elas possuem evidências experimentais que as sustentam. Numerosos estudos em modelos animais têm demonstrado o papel temporário do hipocampo na evocação da memória. Como ilustrado na figura, Kim and Fanselow, 1992 demonstraram que lesões hipocampais causavam déficit na evocação da memória quando o teste era realizado poucos dias (1, 7 e 14) após o aprendizado, mas não quando era realizado em períodos mais remotos (28 dias). Outros estudos que lesionaram ou inativaram farmacologicamente o hipocampo, utilizando diferentes tarefas comportamentais, também demonstraram esse mesmo gradiente temporal. A evocação da memória era inicialmente dependente do hipocampo e com o passar do tempo tornava-se dependente de estruturas corticais (Anagnostaras *et al*, 1999; Haubrich *et al*, 2016; de Oliveira Alvares *et al*, 2012; Pedraza *et al*, 2017a; Sierra *et al*, 2017; Tayler and Wiltgen, 2013).

As estruturas envolvidas com evocação de memórias recentes e remotas também foram avaliadas através da marcação de genes de expressão imediata (Frankland, 2004; Maviel, 2004) ou medidas de atividade metabólica (Bontempi *et al*, 1999) em estudos com roedores. Como pode ser visualizado na **Figura 4**, o hipocampo está mais ativo (cores vermelha e amarela) quando a memória é evocada 5 dias após a aquisição (Figura 4a), estando menos ativo quando uma memória remota (25 dias após) é evocada (Figura 4b); o hipocampo volta a ser mais ativado quando um teste de discriminação contextual é realizado em um período remoto (Figura 4c) (Bontempi *et al*, 1999). Isso reforça a ideia que memórias recentes (Figura 4a) e ricas em detalhes (Figura 4c) dependem do hipocampo para serem evocadas. Adicionalmente, foi demonstrado que anormalidades dos mecanismos de plasticidade do córtex pré-frontal prejudicam a reorganização estrutural da memória, prejudicando a consolidação sistêmica (Frankland *et al*, 2001; Hayashi *et al*, 2004) .

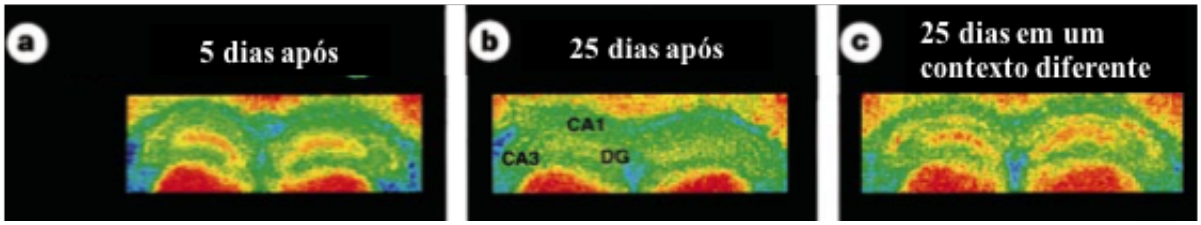

Figura 4 – Representação da atividade hipocampal durante a evocação de uma memória recente (a), remota (b) ou um teste de discriminação contextual 25 dias após a aquisição da memória. As cores vermelha e amarela representam áreas com maior ativação e as cores e azul representam regiões que estão sendo menos ativadas. Significado das abreviaturas: CA1: região 1 do *Cornu Ammonis* hipocampal; CA3: região 3 do *Cornu Ammonis* hipocampal; DG: giro denteado. Figura adaptada de Bontempi et al., 1999

Por outro lado, alguns estudos sugerem que o hipocampo continua sendo recrutado para a evocação de memórias remotas (Broadbent and Clark, 2013; Lehman and Malmberg, 2013) e espaciais (Clark *et al*, 2005; Martin *et al*, 2005; Winocur, 1984; Winocur *et al*, 2005). Também ainda está sob discussão se o grau de aversividade da memória alteraria a dinâmica (isto é, velocidade) com que uma memória se torna independente do hipocampo (Lehman and Malmberg, 2013; Pedraza *et al*, 2017b).

É importante ressaltar que o tipo de memória (contextual ou não) que está sendo avaliado possivelmente influencie o recrutamento do hipocampo. Dessa forma, a teoria da transformação parece se encaixar melhor nos resultados divergentes obtidos até agora. Memórias contextualmente ricas sempre requereriam o hipocampo para serem evocadas, ao passo que memórias com poucos detalhes seriam dependentes de estruturas corticais (Winocur *et al*, 2007; Winocur and Moscovitch, 2011).

Importante mencionar também que durante períodos de repouso e sono é observado a reativação (do inglês, *replay*) dos circuitos neuronais recrutado para o aprendizado. A interferência dessas reativações causa tanto déficits na consolidação sináptica quanto na sistêmica, sugerindo que elas são necessárias para os processos de consolidação (Foster and Wilson, 2006; Nádasdy *et al*, 1999; Wilson and McNaughton, 1994).

1.1.5. Memória de medo

Dentre as diferentes memórias (contextuais, espaciais, de procedimento, etc), a memória de medo é o tipo mais bem estudado. A indução da formação de uma memória de medo é realizada experimentalmente utilizando-se uma tarefa comportamental que envolve um

estímulo aversivo bastante pronunciado, como um choque. A principal vantagem da utilização desse tipo de tarefas é a formação de uma memória duradoura com apenas uma sessão de condicionamento (Curzon *et al*, 2009). Quanto ao tipo de condicionamento, podem ser utilizados tanto o (i) clássico, como o (ii) instrumental. No condicionamento clássico, o animal aprende a associar um estímulo considerado neutro, o estímulo condicionado (do inglês, *conditioned stimulus*, CS), com outro estímulo, aversivo ou apetitivo, que por si só gera uma resposta comportamental, chamado de estímulo incondicionado (do inglês, *unconditioned stimulus*, US). Quando novamente exposto ao CS, o animal gera uma resposta condicionada a esse estímulo, que só é expressa na presença dele. Já no condicionamento instrumental, o animal aprende a reforçar ou inibir um comportamento pela associação dele à um US (Izquierdo *et al*, 2016).

As tarefas comportamentais a que os animais são usualmente expostos durante os experimentos possuem graus diferentes de aversividade. Algumas delas podem gerar ansiedade e estresse, mas não necessariamente medo. O simples fato de um animal ser exposto a um ambiente novo, como na habituação ao campo aberto, pode causar neofobia e, consequentemente, um certo grau de ansiedade. Quando são expostos pela primeira vez ao labirinto aquático, além da neofobia, a água também pode atuar como um estímulo estressor, aumentando assim o grau de aversividade da tarefa. Nesse mesmo *continuum*, tarefas onde um choque elétrico é aplicado nas patas do animal, aumentam ainda mais o grau de aversividade, desencadeando respostas características do comportamento de medo, como a imobilidade ou congelamento (do inglês, *freezing*) (LeDoux, 2014) .

A formação do aprendizado de medo envolve uma circuitaria complexa, requerendo a atividade conjunta de diversas estruturas encefálicas. Na figura 5, está representado, de forma geral, o circuito envolvido na formação da memória de medo de um condicionamento clássico. O hipocampo dorsal, a amígdala basolateral (BLA) e lateral (LA) são essenciais para a codificação e armazenamento das memórias de medo, sendo muito importante as conexões bidirecionais entre essas estruturas (PITKÄNEN *et al*, 2006). A principal forma de entrada e de saída de informações do hipocampo acontece através do córtex entorrinal. Este, por sua vez, manda projeções para outras regiões corticais que estão envolvidas nos mecanismos de controle da atividade do hipocampo e da amígdala; dentre elas ressaltam-se algumas subáreas do córtex medial pré-frontal, como: o córtex cingulado anterior, o córtex pré-límbico e o córtex infralímbico. No complexo amigdaloide, ou amígdala, as informações processadas na BLA são enviadas para a amígdala central (CE), que é um dos núcleos de saída dessa estrutura encefálica

(Izquierdo *et al*, 2016). A CE projeta-se (i) para o córtex medial pré-frontal, envolvido com a formação da memória, (ii) para os núcleos do hipotálamo lateral, que são encarregados pelo componente simpático da resposta de medo, e (iii) para a substância cinzenta periaquedutal, que é responsável pela resposta comportamental de imobilidade (LeDoux, 2007).

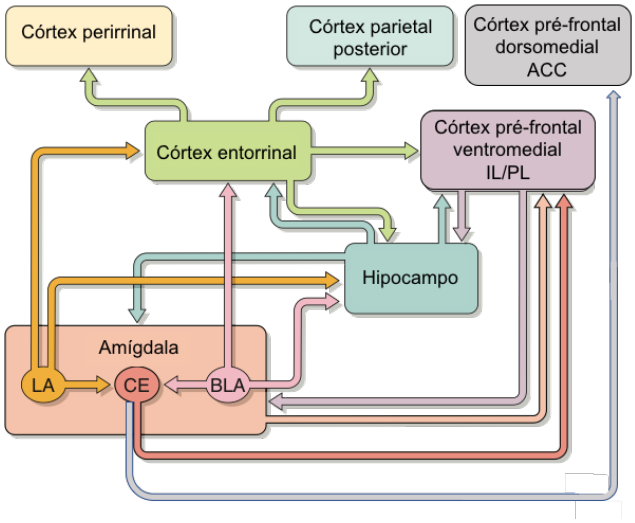

Figura 5 – Estruturas encefálicas envolvidas com a formação da memória de medo. Adaptado de Izquierdo et al., 2016.

Contudo, existem algumas particularidades na circuitaria recrutada dependendo de qual estímulo é utilizado como condicionado. O hipocampo é principalmente recrutado no CAC, ao passo que na tarefa de condicionamento aversivo à dica (do inglês, *cue fear conditioning*, onde uma luz ou som é associado ao contexto) a LA parece ser essencial para a associação entre o CS e o US (Curzon *et al*, 2009).

Nesse trabalho utilizamos unicamente a tarefa de CAC, explorando essencialmente os mecanismos de plasticidade recrutados para a formação da memória.

1.2. Anatomia e circuitaria hipocampal

Ao longo do eixo longitudinal hipocampal (ou eixo ântero-posterior) são encontradas diferenças na conectividade com outras estruturas, expressão gênica e funcionalidade (Strange *et al*, 2014) **(Figura 5**, esquerda**)**. O hipocampo dorsal, interliga-se principalmente com regiões encefálicas associadas com cognição, ao passo que o hipocampo ventral está mais associado a regiões que contribuem para as reações emocionais (Fanselow and Dong, 2010). Além disso, a região intermediária, entre o hipocampo dorsal e ventral, também pode ser diferenciada das

outras regiões. Estudos anatômicos e de expressão gênica (Thompson *et al*, 2008) sugerem que o hipocampo intermediário é funcionalmente mais parecido com o hipocampo dorsal (Ferbinteanu *et al*, 2006; Rudy and Matus-Amat, 2005). Alguns autores inclusive sugerem que experimentos farmacológicos demonstrando funções cognitivas similares entre o hipocampo dorsal e ventral podem ter atingido parte do hipocampo intermediário ao invés do ventral (Fanselow and Dong, 2010).

Apesar das diferenças funcionais entre o hipocampo dorsal, intermediário e ventral, a circuitaria trissináptica é encontrada ao longo de todo o eixo ântero-posterior. O circuito trissináptico hipocampal **(Figura 6**, direita**)** foi descrito, por volta de 1900, por Ramón y Cajal. De forma simplificada, os axônios de neurônios do EC entram no giro denteado (DG, do inglês, *dentate gyrus*) através da via perfurante, fazendo sinapse nas células granulares do DG, sendo essa considerada a primeira sinapse do circuito. Na sequencia, os axônios das células granulares, chamados de fibras musgosas, fazem sinapses com as células piramidais da região CA3 (CA, do grego, *Cornu Ammonis*) do hipocampo (segunda sinapse). Esses neurônios projetam seus axônios através da colateral de Schaffer para as células piramidais da região CA1 (terceira sinapse). Os axônios de CA1 deixam o hipocampo e projetam-se principalmente para o subículo (Brady *et al*, 2012). É importante ressaltar que apesar de todas as sinapses supracitadas serem glutamatérgicas, entre as fibras musgosas e CA3 ocorre um tipo de plasticidade que é independente da ativação dos NMDARs, sendo o aumento da eficácia sináptica causado tanto por alterações pré-sinápticas quanto pós-sinápticas (Nicoll and Malenka, 1995). Dessa forma, pode ocorrer tanto plasticidade Hebbiana quanto não Hebbiana na sinapse entre as fibras musgosas e CA3 (Urban and Barrionuevo, 1996).

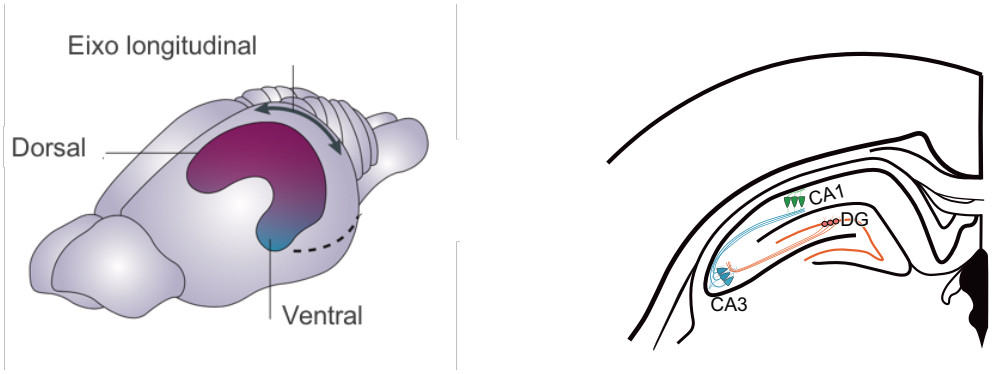

Figura 6 – Desenho esquemático representando o eixo longitudinal do hipocampo (esquerda) e o circuito trissináptico hipocampal (direita). Imagem à esquerda adaptada de Strange et al., 2014 e imagem à direita retirada de Izquierdo et al., 2016.

1.3. Receptores glutamatérgicos

A ação do glutamato ocorre através da sua ligação aos receptores glutamatérgicos, que dividem-se em ionotrópicos (do inglês, *ionotropic glutamate receptors,* iGluRs) e metabotrópicos (do inglês, *metabotropic glutamate receptors*, mGluRs). Classicamente, os iGluRs são canais iônicos, ao passo que os mGluRs são acoplados à proteína G.

Os iGluRs são tetraméricos, isto é, são formados pela junção de 4 subunidades individuais. A heterogeneidade das combinações de subunidades proporciona diferenças na funcionalidade dos receptores. Os iGluRs são divididos em três famílias: AMPAR, NMDAR e KAR (do inglês, *kainate receptor*). A importância dos receptores AMPA e NMDA são bem elucidadas na formação de memórias e nos fenômenos de plasticidade sináptica Hebbiana (Brown *et al*, 2010; Tse *et al*, 2011; Voglis and Tavernarakis, 2006). A ligação do glutamato nos receptores causa primeiramente a abertura dos AMPAR, permitindo a entrada de cátions que despolarizam parcialmente a membrana. Essa despolarização parcial também é responsável pela saída do íon Mg^{2+} que bloqueia o poro do receptor NMDA permitindo então a sua abertura, o que aumenta ainda mais a permeabilidade de cátions. A entrada do cálcio, principalmente pelos NMDARs, desencadeia a ativação de diversas vias de sinalização intracelular que estão envolvidas com os fenômenos plásticos que foram melhor detalhadas anteriormente (Kandel *et al*, 2000).

Os AMPARs são compostos por subunidade classificadas como GluA1-GluA4 **(Figura 6)**. Eles normalmente são impermeáveis ao cálcio (do inglês, *calcium impermeable* AMPAR, CI-AMPAR). Contudo, receptores que não contém a subunidade GluA2, passam a ser permeáveis (do inglês, *calcium permeable* AMPAR, CP-AMPAR). Os CP-AMPA são expressos transientemente nos estados iniciais da plasticidade sináptica (Plant *et al*, 2006), sendo importantes para a indução desse tipo de plasticidade (Clem and Huganir, 2010). Depois, eles são substituídos pelos CI-AMPA, que são importantes para a manutenção da plasticidade sináptica (Man, 2011).

Os receptores NMDA são compostos por subunidades que podem ser dos tipos GluN1, GluN2 e GluN3, sendo a GluN1 constitutiva. A subunidade GluN2 pode ser subdividida em A, B, C ou D e a subunidade GluN3 em A e B, como pode ser visto na figura 6 (Brady *et al*, 2012; Paoletti *et al*, 2013). Os NMDARs possuem pelo menos seis sítios de ligantes endógenos, sendo que o glutamato se liga na subunidade GluN2 e os co-agonistas (p.ex. glicina) nas subunidades GluN1 e GluN3 (Matt and Hell, 2014; Baez et al., 2018). Para causar a inativação dos

NMDARs, no presente estudo foi utilizado o AP5 (do inglês, *(2R)-amino-5-phosphonopentanoate*) que é uma fármaco ligante do mesmo sítio de ligação do glutamato, sendo, portanto, classificado como um antagonista competitivo do NMDAR.

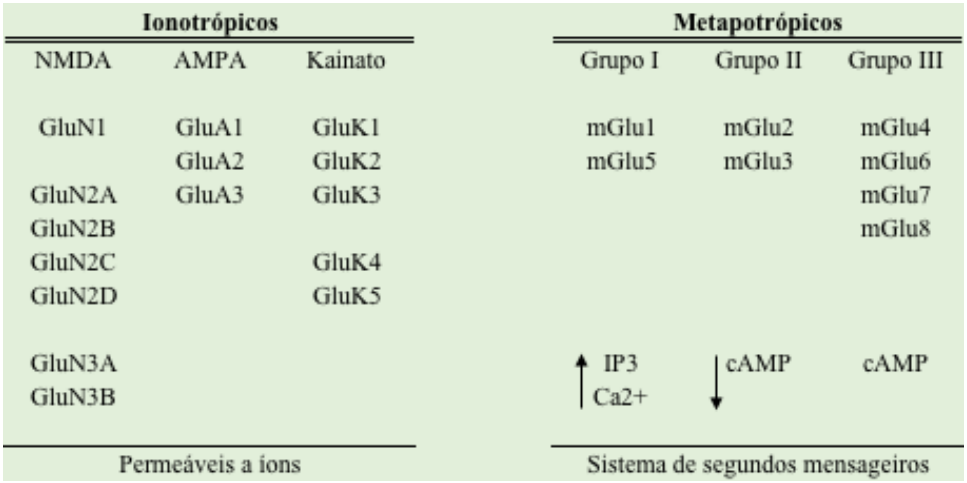

Figura 7 – Quadro esquemático representado os tipos de receptores glutamatérgicos, suas subunidades e vias de sinalização. Adaptado de Brady et al., 2012.

Os KARs possuem subunidades GluK1-GluK5 **(Figura 6)**. Apesar de existirem estudos sobre o seu tráfego nos espinhos dendríticos (Coussen, 2009) e de já ter sido demonstrada a importância deles em fenômenos de plasticidade sináptica (Bortolotto *et al*, 1999; Petrovic *et al*, 2017), os KARs são os receptores ionotrópicos glutamatérgicos que tem sua função menos esclarecida.

Os mGluRs são divididos em grupo I, II e III. Os mGluRs do grupo I (mGluR1 e mGluR5) ativam proteínas G estimulatórias (Gq) iniciando vias de sinalização que envolvem fosfolipase C/inositol 1,4,5 trifosfato/diacilglicerol (do inglês*, phospholipase C*, PLC; *inositol 1,4,5-triphosphate*, IP3; *diacylglycerol*, DAG). O aumento dos níveis de cálcio intracelular, pode ativar CaM (do inglês, *calmodulin*), CaMK (do inglês, *Ca^{2+}/CaM-dependent kinases*) e vias de sinalização subsequentes que são importantes para memória e para a LTP. Receptores dos grupos II e III reduzem os níveis de AMPc (monofosfato cíclico de adenosina), por sinalizarem através de uma proteína G inibitória (Gi). Grupo II engloba os receptores mGluR2 e mGluR3, ao passo que o grupo III inclui os mGluR4, mGluR6, mGluR7 e mGluR8 (Willard and Koochekpour, 2013).

1.4. Plasticidade Hebbiana

Uma das primeiras ideias de como uma experiência comportamental causava alterações nos neurônios foi proposta por Ramón y Cajal, ainda no século XIX. Ele predisse que as conexões (depois identificadas como sinapses) entre os axônios e as protusões (espinhos dendríticos) eram modificadas pela experiência (Josselyn *et al*, 2015b). Essas ideias foram aperfeiçoadas por Hebb, que postulou que atividade coincidente entre o neurônio pré-sináptico e o pós-sináptico fortaleceria a conexão sináptica entre eles (Morris, 1999). Dessa forma, o fortalecimento das conexões sinápticas poderia ser responsável pela formação de conjuntos de neurônios que estariam associados e seriam responsáveis, por exemplo, pelo aprendizado e pela consequentemente evocação da memória (Josselyn *et al*, 2015b). Atualmente, denomina-se plasticidade Hebbiana a atividade coordenada entre o neurônio pré-sináptico e pós-sináptico.

Em suporte a teoria de Hebb, Bliss e Lomo (1973) descobriram a LTP, que é assim denominada devido ao aumento duradouro da eficácia sináptica entre o neurônio pré-sináptico e pós-sináptico. O aumento da eficácia sináptica induzido pela ativação dos NMDARs é inicialmente sustentado pela fosforilação e inserção de AMPARs. Posteriormente, a ativação de vias de sinalização intracelular leva a indução de síntese de proteínas necessárias para a manutenção da potenciação. Contrapondo o aumento da eficácia sináptica, também pode ser observada a diminuição, chamada de depressão de longa duração (LTD, do inglês, *long-term depression*) (Kandel *et al*, 2000). Existem diferentes protocolos para indução da LTP ou LTD, que vão depender da sinapse que está sendo estudada e das influências extrínsecas (nível de inibição sináptica, neuromoduladores, hormônios, etc) que podem modular a facilidade com que a plasticidade sináptica será desencadeada (Abraham and Williams, 2008). De forma geral, a estimulação elétrica de alta frequência nos axônios das diferentes regiões hipocampais (DG, CA3, CA1) gera um aumento da eficácia sináptica (LTP), ao passo que protocolos de baixa frequência geram diminuição da força sináptica nas sinapses dessa mesma estrutura (Bear and Malenka, 1994; Malenka and Bear, 2004). Por exemplo, para a indução da LTP nas células piramidais de CA1 costuma-se utilizar estimulação de alta frequência (HFS, do inglês, *high frequency stimulation*; estimulação contínua de 100 Hz por 1 segundo) ou estimulação *burst* (4 pulsos de 100 Hz repetidos a cada 200ms). Já para a indução de LTD nessas mesmas células utiliza-se estimulação de baixa frequência (LFS, do inglês, *low frequency stimulation*; 1-3 Hz) por um período prolongado (10-15 minutos) (Bliss and Cooke, 2011; Malenka and Bear, 2004).

Além destes, existem protocolos de indução que prezam pela relação temporal entre o disparo do neurônio pré-sináptico e a despolarização do neurônio pós-sináptico, denominado *plasticidade dependente do tempo de disparo* (STDP, do inglês, s*pike-timing dependent plasticity*). Esses são considerados os protocolos que mais se aproximam dos fenômenos plásticos que provavelmente ocorrem fisiologicamente. Quando o estímulo pré-sináptico é realizado antes do pós-sináptico e o tempo entre os estímulos é curto, é observado aumento da força sináptica. Por outro lado, quando a atividade do neurônio pós-sináptico ocorre antes do pré-sináptico, é observada uma diminuição da força sináptica (Buonomano and Carvalho, 2010).

Desde que estudos farmacológicos mostraram a necessidade de NMDAR para a formação de memórias dependentes de hipocampo (Morris et al., 1986) e que trabalhos eletrofisiológicos demonstraram o recrutamento do mesmo receptor para a indução de LTP na via das colaterais de Schaffer para CA1, tem-se tentado demonstrar um elo direto entre o aprendizado e o aumento da força sináptica. Dessa forma, tem sido proposto que as alterações na força sináptica atuam como um dos possíveis substratos neurobiológicos para o armazenamento de informações (Tonegawa *et al*, 2015).

O estudo de Nabavi et al., 2014 foi um dos que tentou evidenciar uma relação causal entre memória e alterações na força sináptica. Nesse trabalho, ratos associaram o choque nas patas com a estimulação optogenética da amígdala lateral. Posteriormente, quando uma LTP foi optogeneticamente induzida, os animais evocaram a memória de medo; por outro lado a indução de uma LTD prejudicou a evocação (Nabavi *et al*, 2014). Contudo, esse trabalho não demonstrou que a plasticidade sináptica foi induzida especificamente nas células recrutadas para a formação da memória. Um trabalho do grupo do Tonegawa complementou esse estudo citado anteriormente, demonstrando que o aprendizado causa um aumento da força sináptica (isto é, a ativação do neurônio pré-sináptico causa um aumento no potencial excitatório pós-sináptico) especificamente nas células do giro denteado do hipocampo que foram recrutadas para o aprendizado (Ryan *et al*, 2015).

1.5. Plasticidade homeostática e metaplasticidade

Mudanças plásticas causam alterações no número e na força das sinapses, fazendo-se necessário o uso de mecanismos de estabilização, ou mecanismos homeostáticos, para manter a atividade neuronal dentro de uma faixa fisiológica (Turrigiano, 2012). Modelos

computacionais predizem que sem algum mecanismo de estabilização do peso sináptico (isto é, eficácia com que um neurônio se comunica com o outro) ou das frequências de disparos de potenciais de ação, a atividade sináptica ficaria descontrolada (Keck *et al*, 2017). Dessa forma, os mecanismos homeostáticos são de extrema importância para o controle e manutenção da atividade encefálica como um todo. Para um mecanismo de plasticidade ser considerado homeostático ele deve regular determinado parâmetro dentro de um valor estabelecido como 'normal' (Turrigiano, 2012). Dentre os principais mecanismos de estabilização homeostática, estão: (i) escalonamento sináptico, (ii) controle da inibição sináptica, (iii) modulação dos espinhos dendríticos, e (iv) alteração do limiar de indução da plasticidade sináptica causada por atividade sináptica prévia (Keck *et al*, 2017).

Não apenas a plasticidade sináptica pode mediar o aprendizado. Mecanismos de plasticidade homeostática, como a metaplasticidade, também podem dar suporte ao aprendizado. Denominamos metaplasticidade quando a indução da plasticidade sináptica é alterada por modificações plásticas anteriores **(Figura 8)**. O prefixo 'meta', do grego 'além' ou 'acima', é usado para indicar uma forma de plasticidade sináptica de alta ordem, a plasticidade da plasticidade sináptica (Abraham and Bear, 1996). Uma atividade desencadeadora de metaplasticidade pode modificar a sinapse de forma que o limiar de indução (ou outras características da plasticidade: intensidade, direção, ocorrência ou não) de plasticidades sinápticas posteriores seja alterado. Neste trabalho avaliamos se a metaplasticidade induzida pelo aprendizado alterou a excitabilidade neuronal.

Alguns trabalhos prévios demonstraram que a excitabilidade neuronal pode ser mantida aumentada por até 5 dias após o aprendizado em neurônios piramidais de CA1 (Moyer *et al*, 1996). Sugere-se que a hiperexcitabilidade seja mediada pelo aumento da PKC (do inglês, *protein kinase C*) e da atividade de ERK1 e ERK2 (do inglês, *extracellular-signal-regulated-kinase 1 e 2*) (Cohen-Matsliah *et al*, 2007). Além disso, aumento dos níveis de CREB tornam os neurônios mais excitáveis e propícios para serem recrutados para o aprendizado associativo (Yiu *et al*, 2014; Zhou *et al*, 2009).

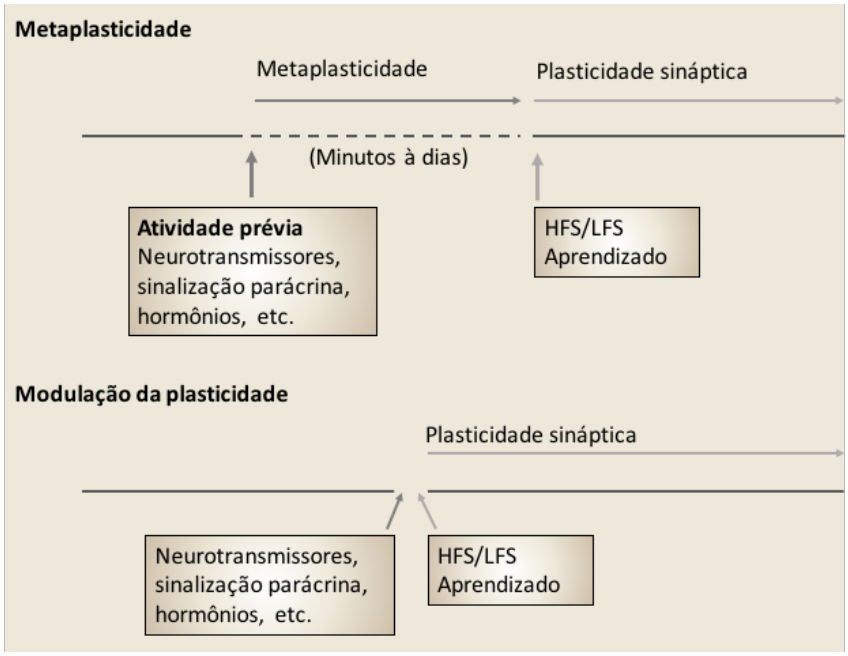

Figura 8 – Representação esquemática de como a metaplasticidade influencia a indução da plasticidade sináptica subsequente (acima) e como diferentes fatores atuam no momento de indução da plasticidade sináptica (abaixo). Adaptado de Abraham, 2008.

1.6. Aquisição de memórias independentes de NMDARs

As informações contidas nessa subseção foram adaptadas do artigo de revisão publicado pela autora, intitulado: "Can Previous Learning Alter Future Plasticity Mechanisms?". Por ele conter informações aquém das abordadas nessa tese, encontram-se resumidas a seguir apenas as informações consideradas mais relevantes. O artigo completo encontra-se no apêndice.

A importância dos NMDARs na formação de memórias tem sido demonstrada por vários estudos em diferentes tarefas comportamentais (Alaghband and Marshall, 2013; Cain *et al*, 1996; Fanselow *et al*, 1994; Faust, 2013; Kim *et al*, 1991; Morris, 1989; Morris *et al*, 1986; Vianna *et al*, 2001; Young *et al*, 1994). Contudo, em algumas situações, o aprendizado subsequente ocorre mesmo com o bloqueio desses receptores (Bannerman et al, 1995; Sanders & Fanselow, 2003; Roesler et al, 1998; Tayler et al, 2011; Wiltgen et al, 2010; 2011). A primeira demonstração desse fenômeno foi realizada pelo grupo do Morris em um clássico experimento realizado no labirinto aquático, que ele mesmo desenvolveu. O experimento ficou conhecido como *downstairs-usptairs* porque os animais foram expostos a dois labirintos aquáticos, um localizado em uma sala *downstairs* e outro em uma sala *upstairs*, salas essas que possuíam diferentes dicas contextuais. Foi observado que animais que já haviam aprendido a

tarefa previamente, não necessitavam os NMDARs para o aprendizado posterior (Bannerman et al., 1995). Contemporaneamente, Saucier & Cain (1995) demonstraram que a familiaridade com a tarefa era suficiente para tornar o próximo aprendizado independente dos NMDARs. A presença desse fenômeno também foi evidenciada na formação de memórias aversivas. Roesler et al (1998) mostraram que a pré-exposição ou o pré-treino na esquiva inibitória preveniam o efeito amnésico do antagonista dos NMDARs (APV). Em concordância, outros trabalhos utilizando o condicionamento aversivo contextual indicam que os NMDARs não são recrutados quando os animais possuem uma experiência prévia nessa mesma tarefa (Hardt et al., 2009; Sander & Fanselow, 2003; Tayler et al., 2011). Assim, sugere-se que uma experiência prévia pode modificar a forma como o próximo aprendizado será adquirido, caracterizando um fenômeno de metaplasticidade. Dessa maneira, a aquisição do aprendizado subsequente seria mediada por diferentes mecanismos de plasticidade, sem a necessidade dos NMDARs.

Alguns trabalhos têm sugerido que os CP-AMPARs poderiam mediar a plasticidade sináptica dos aprendizados independentes de NMDARs (Wiltgen et al, 2010; Clem et al, 2008). Contudo, essa questão não está muito clara na literatura e outros receptores podem ter um papel tão ou mais importante do que os CP-AMPARs. Além disso, outros estudos têm sugerido que o aprendizado prévio na mesma tarefa possa modificar a estrutura encefálica envolvida com a formação do aprendizado subsequente (Cammarota et al., 2005; de Hoz & Martin, 2014; Wang et al., 2012). Dessa forma, para um aprendizado ser independente de NMDAR ele deve atender a dois critérios principais (i) acontecer na mesma tarefa comportamental ou no mesmo contexto do que o aprendizado prévio e (i) ser mediado pela mesma estrutura encefálica utilizada para a aquisição do primeiro aprendizado. Se os aprendizados são muito diferentes entre si, como o aprendizado do labirinto aquático seguido pelo CAC, os NMDARs ainda são recrutados (Wiltgen et al., 2011). Se os aprendizados não requerem a mesma estrutura encefálica, não existe a possibilidade de inferir algo sobre como a metaplasticidade induzida pelo primeiro aprendizado influencia no aprendizado subsequente. Sendo assim, o melhor modelo para explicar como a metaplasticidade induzida pela primeira experiência influencia no aprendizado seguinte seria através da reutilização dos mesmos neurônios, observando-se uma sobreposição entre as populações neuronais recrutadas para ambos os aprendizados. Além disso, não se sabe ao certo por quanto tempo a metaplasticidade consegue influenciar no próximo aprendizado, apesar de já ter sido observados que ele pode ocorrer de forma independente dos NMDARs até trinta dias após a aquisição da memória anterior (Wiltgen et al, 2011).

Dessa forma, ainda é elusivo de que forma e por quanto tempo a metaplasticidade induzida pelo primeiro aprendizado influencia na aquisição do aprendizado subsequente. No presente trabalho, buscamos responder essas perguntas. Sendo nossas hipóteses as seguintes:

1.6.1. Capítulo I

A metaplasticidade induzida pelo primeiro aprendizado aumenta a excitabilidade intrínseca dos neurônios favorecendo a codificação de novas memórias via ativação de receptores metabotrópicos glutamatérgicos (mGluR).

1.6.2. Capítulo II

Aprendizados muito afastados temporalmente (memória remota) *não* são mais influenciados pelos mecanismos metaplasticidade induzidos pelo primeiro aprendizado.

2. OBJETIVOS GERAIS

Determinar (i) de que forma e (ii) por quanto tempo os mecanismos de metaplasticidade, induzidos pelo primeiro aprendizado influenciam em um aprendizado subsequente recente ou remoto.

2.1. Objetivos específicos do capítulo I

2.1.1. Verificar se o aprendizado da tarefa de condicionamento aversivo ao contexto causa um aumento da excitabilidade intrínseca, que dura pelo menos dois dias, nos neurônios recrutados para o aprendizado.

2.1.2. Avaliar se os neurônios recrutados para o primeiro aprendizado são mais propensos a serem utilizados para o segundo aprendizado.

2.1.3. Verificar se o primeiro aprendizado é mediado por NMDARs.

2.1.4. Avaliar se o aprendizado subsequente é independente dos NMDARs nas presentes condições experimentais.

2.1.5. Determinar se os mGluRs do tipo I são necessários para a formação do primeiro aprendizado.

2.1.6. Verificar se os mGluRs do tipo I são recrutados para o aprendizado subsequente.

2.2. Objetivos específicos do capítulo II

2.2.1. Avaliar se a memória remota pode ser evocada quando ambos hipocampo dorsal e ventral são inativados.

2.2.2. Determinar se a inativação do córtex cingulado anterior prejudica a formação de um aprendizado subsequente a uma memória que já foi consolidada sistemicamente.

2.2.3. Verificar se a inativação do hipocampo dorsal prejudica a formação do aprendizado subsequente a uma memória remota.

2.2.4. Avaliar se o aprendizado subsequente a uma memória é prejudicado pela inativação do hipocampo ventral.

2.2.5. Determinar se a inativação concomitante do hipocampo dorsal e ventral causa um déficit na formação do aprendizado subsequente a uma memória remota.

2.2.6. Verificar se as pré-exposições modificam o mecanismo de plasticidade sináptica que vai ser recrutado para o primeiro aprendizado.

2.2.7. Avaliar se a metaplasticidade induzida pelo primeiro aprendizado ainda influencia no aprendizado subsequente quando o intervalo entre eles é aumentado para quarenta dias.

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4. CAPÍTULO II

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Hippocampal plasticity mechanisms mediating experience-dependent learning change over time

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ABSTRACT

The requirement of NMDA receptor (NMDAR) activity for memory formation is well described. However, the plasticity mechanisms for memory can be modified by experience, such that a future similar learning becomes independent of NMDARs. This effect has often been reported in learning events conducted with a few days interval. In this work, we asked whether the NMDAR-independency is permanent or the brain regions and plasticity mechanisms of experience-dependent learning may change over time. Considering that contextual memories undergo a gradual reorganization over time, becoming progressively independent from the hippocampus and dependent upon cortical regions, we investigated the brain regions mediating a new related learning conducted at a remote time-point, when the first memory was already cortically established. First, we demonstrated that anterior cingulate cortex was not able to support a learning subsequent to a previous systems-level consolidated memory; it did require at least one functional subregion of the hippocampus (ventral or dorsal). Moreover, after replicating findings showing that a few days interval between trainings induces a NMDAR-independent learning, we managed to show that a learning following a longer interval once again becomes dependent on NMDARs in the hippocampus. These findings suggest that while the previous memory grows independent from the hippocampus over time, an experience-dependent learning following a systems-consolidated memory once again engages the hippocampus and a NMDAR-dependent plasticity mechanism.

1. Introduction

Decades of research have led to significant advances in our understanding of the brain mechanisms underlying learning and memory. However, this knowledge was built almost exclusively on experimental models employing *naïve animals*, who typically undergo a single learning experience in their whole life. This is problematic since in real-life situations we are continuously forming new memories, which may involve important neurobiological adaptations that are poorly understood. Consistent evidence begins to emerge from a handful of studies pointing to a decisive role of previous experiences in how subsequent learning is encoded (for review see Crestani & Quillfeldt, 2016).

Memories and synapses are dynamic in nature and capable of strengthening and weakening depending on behavioral tasks and stimulation protocols (Dudek & Bear, 1993; Kealy & Commins, 2010). It is well established that memory consolidation involves NMDAR-mediated synaptic plasticity (Cercato et al., 2014; Fanselow & Kim, 1994; Huerta, Sun, Wilson, & Tonegawa, 2000; Inglis, Martin, & Morris, 2013; Kim, DeCola, Landeira-Fernandez, & Fanselow, 1991; Morris et al., 1986, 2013; Morris, 1989; Shimizu, 2000; Tsien, Huerta, & Tonegawa, 1996). However, synapses are capable of metaplasticity, whereby prior exposure (either behavioral or electrophysiological) can alter the plasticity of synapses (Bienenstock & Munro, 1982). In other words, prior experience may cause modifications in the plasticity mechanisms that will then support a following learning. If animals have previously learned a task, the subsequent memory acquisition of a similar task will not depend on NMDAR recruitment. This phenomenon was demonstrated in different hippocampus-dependent behavioral tasks, such as the water maze (Bannerman, Good, Butcher, Ramsay, & Morris, 1995; Saucier & Cain, 1995; Saucier, Hargreaves, Boon, Vanderwolf, & Cain, 1996), contextual fear conditioning (Caramanos & Shapiro, 1994; Inglis et al., 2013; Sanders & Fanselow, 2003; Tayler et al., 2011; Wiltgen et al., 2010, 2011) and inhibitory avoidance (Cammarota, Bevilaqua, Köhler, Medina, & Izquierdo, 2005; Roesler et al., 1998). Furthermore,

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it was shown that NMDAR-independent learning only occurs when animals are subsequently trained in the same task (Wiltgen, Wood, & Levy, 2011), requiring at least a certain degree of similarity between contexts (Tayler et al., 2011). Behavioral protocols used to evince and study NMDAR-independent learning usually have an interval of a few days between subsequent trainings. To this day, however, longer intervals have not been evaluated.

Another phenomenon that may take place with longer training-test intervals is systems consolidation, in which memories undergo a gradual brain reorganization over time (Frankland & Bontempi, 2005). With the assistance of activity-related genes (c-fos, Arc or Zif268, see Barry et al., 2016 for differences between them) or metabolic imaging activity ([^{14}C]2-deoxyglucose), it was shown that over intervals of several weeks, memory retrieval depends progressively less on the hippocampus while, in parallel, an increasing engagement of the cortical areas takes place (Bontempi, Laurent-Demir, Destrade, & Jaffard, 1999; Frankland, Bontempi, Talkton, Kaczmarek, & Silva, 2004; Kitamura et al., 2017; Maviel, 2004). Evidence points to the anterior cingulate cortex (ACC) as a repository of remote memories necessary to support their retrieval in a later moment (Bontempi et al., 1999; Cain, Saucier, Hall, Hargreaves, & Boon, 1996; Ding, Teixeira, & Frankland, 2008; Frankland, 2004; Haubrich et al., 2016; Kitamura et al., 2017; Lopez et al., 2012; Maviel, 2004; Teixeira, Pomedli, Maei, Kee, & Frankland, 2006; Weible, Rowland, Monaghan, Wolfgang, & Kentros, 2012). Despite the wealth of studies demonstrating the dependence of remote memories on the ACC, it remains unclear whether this area is also required to support the acquisition of a similar learning experience at a remote time point.

Thus, our main aim here was to investigate whether a hippocampus-independent remote memory can affect the acquisition of a subsequent memory, checking for the neuroanatomical and neurochemical changes verified. In the first experiment, we analyzed whether the retrieval of remote memory was effectively independent from the hippocampus, while in the second one, we investigated the ACC and HPC engagement in the encoding of a learning event subsequent to a memory already consolidated at the systems-level. Finally, we evaluated the plasticity mechanisms that mediate this experience-dependent learning by testing NMDAR dependence or independence either at a recent (3-day), or a remote (40-day) time point.

2. Material and methods

2.1. Animals

Male Wistar rats weighing 250–350 g from our University breeding colony (CREAL/UFRGS) were used. Animals were housed in plastic cages, five per cage, under a 12 h light/dark cycle and at constant temperature of 21 ± 1 °C, with water and food ad libitum. All experiments were conducted in accordance to local animal care guidelines (Brazilian Federal Law 11,794/2008) and approved by the Ethics in the Use of Experimental Animals Committee of Federal University of Rio Grande do Sul (CEUA, Project UFRGS #28,277).

2.2. Behavioral procedure - contextual fear conditioning (CFC)

To create a learning event subsequent to a previous memory, rats underwent fear conditioning in context A followed by context B.

All experiments (with the exception of Experiment 1A) consisted of pre-exposure to the grid floors (day 1 and 2), training in context A (day 3), test in context A (day 4), training in context B (day 6 or day 43) and test in context B (day 7 or 44) – as described in detail below (see also figures schematics).

For the *recent memory* condition, training in context B was conducted 3 days after training in context A while in the *remote memory* condition, context B training was conducted 40 days later. Testing was always performed one day following training.

Each conditioning chamber was placed in a different room with constant fan background noise. All chambers consisted of the same grid floor: parallel 0.1 cm caliber stainless copper bars spaced 1.0 cm apart and were cleaned with 70% ethanol. The chamber where animals were *pre-exposed* was a rectangular beige Plexiglas box (20 × 50 × 22 cm). *Context A* was a square white Plexiglas box (22 × 22 × 25 cm) while *context B* consisted of a circular polyvinyl chloride box with black and white vertical stripes (diameter: 25 cm, height: 22 cm) and vanilla scent was added to the room; some drops of scent were put in a cotton inside a petri dish that was in a bench near to where animals were conditioned. The only similarity between context A and B was the grid floor.

Animals were pre-exposed to the grid floor for 10 min each day for two days prior to the contextual fear conditioning training which took place on day 3. During the training session, animals were habituated to the context (A or B) for a 3-min baseline period, and then received two 2-s, 0.5 mA footshocks separated by a 30-s interval. They were kept in the conditioning environment for an additional 30-s before they were returned to their homecage. Test sessions consisted of measuring the animal's freezing response during a 4 min re-exposure to the previously trained context.

During the training and test sessions freezing behavior was recorded by stopwatch minute by minute by an experienced observer, blind to the treatment groups. Freezing behavior was defined as the absence of all movements except those related to breathing, and expressed as percentage of total session time.

In the experiment 1A (Experiment 1A), rats were re-tested in context A 40 days after training, rather than trained in context B. This experiment was performed to evaluate whether in our protocol remote memory retrieval was hippocampus-independent.

2.3. Stereotaxic surgery and cannulation

Animals were anesthetized with a ketamine and xylazine association (75 and 10 mg/kg, respectively) infused intraperitoneally. Guide cannulae (22 gauge) were implanted bilaterally at dHPC, vHPC or ACC positioned just 1.0 mm above the target brain region (according to Paxinos & Watson, 1998). Cannulae were positioned at the following coordinates with respect to Bregma (mm): −4.0 A/P, ± 3.0 M/L, −1.6 D/V (from brain surface) for CA1 region of the dHPC; −6.0 A/P, ± 5.0 M/L, −6.5 D/V, for CA1 region of the vHPC; +2.7 A/P, ± 0.5 M/L, −1.5 D/V, for ACC. Rats were allowed to recover for 6–7 days prior to behavioral testing. Animals were injected three times subcutaneously with antibiotic (tylosin – 1 mg/kg) and anti-inflammatory drug (meloxicam - 1.5ml/kg), once immediately preceding the surgery and again once daily, for two days following the surgery.

2.4. Drugs and infusion

Fifteen minutes prior to re-test on context A (Experiment 1A), training on context A (Experiment 2A) or training on context B (all others experiments, also including Experiment 2A) drugs (muscimol or AP5) or vehicle were infused into the target region with a 27 gauge injection cannula. Bilateral infusions were administered simultaneously using a two-syringe micropump. In 4-cannulae experiments, one side of a brain region (e.g., right dHPC) was infused simultaneously with the contralateral side of the other brain area (left ACC), and vice versa. The injectors were left in place for 30 s after the end of the infusion to allow for diffusion. The rats were then returned to their homecage until testing.

Muscimol (Sigma-Aldrich), a $GABA_A$ receptor agonist, was dissolved in a phosphate-buffered saline (PBS) solution to a concentration of 1 μg/μl and it, or its vehicle, was infused into the target brain region at a slow rate (20 μl/h) in a volume of 0.5 μl/hemisphere, 15 min prior to CFC training in context B. The muscimol infusion was used to suppress neuronal firing, thereby temporarily inactivating brain regions of interest.

AP5 (Sigma-Aldrich), an NMDAR antagonist, was diluted in phosphate-buffered saline solution to a concentration of 5 μg/μl and it, or its vehicle, was infused locally into the target brain region at a slow rate (20 μl/h) in a volume of 1.0 μl/hemisphere, 15 min prior to CFC training in context A or B.

2.5. Histology

Following the completion of the behavioral experiments, cannulae placements were verified by injecting 0.5 or 1 μl (the same volume of the drug infused) of methylene blue through the guide cannula. The brains were dissected and fixed in 10% formaldehyde in order to verify cannulae placement under low magnification (4×). If the dye was not observed in the proper place, behavioral data from that rat was excluded from analyses. Cannula placements for each experiment are represented in the supplementary material.

2.6. Statistical analysis

After checking for normality (Kolmogorov-Smirnov Test), data was analyzed by a 2 × 2 mixed-model ANOVA considering as a repeated measure (within measure) only one of the factors, i.e. the context/time that animals were tested (context A vs. context B). The group factor (veh vs. drug) was considered a between measure. Student-Newman-Keuls was used as a post hoc test and significance was set to $P < 0.05$, using Statistica version 7.

3. Results

3.1. Previously acquired, cortically dependent remote memory does not support the acquisition of a similar learning experience

Hippocampal dependency of memory retrieval is usually tested by dorsal hippocampus inhibition (Anagnostaras, Maren, & Fanselow, 1999; Frankland, 2004; Kim & Fanselow, 1992; Maviel, 2004), despite the fact that some have proposed a potential regional compensation within the hippocampus, between dorsal and ventral division when inactivations are performed separately (de Hoz & Martin, 2014; Keinath et al., 2014; Wang, Finnie, Hardt, & Nader, 2012). In light of this possibility, we first tested whether retrieval of remote memory is independent of the entire hippocampus (dPHC + vHPC) infusing the $GABA_A$ agonist muscimol directly into hippocampus. Animals were trained in context A and tested in the same context 40 days later (see Materials and Methods for details). A 2 × 2 mixed-model ANOVA showed that inactivation of dHPC + vHPC before testing at a remote time point did not impair memory retrieval. No significant effects of *drug* ($F_{(1,12)} = 0.0442$, $P = 0.8370$) or *time* ∗ *drug* interaction were observed ($F_{(1,12)} = 0.0000$, $P = 0.9960$) (Fig. 1A), indicating that remote memory retrieval does not depend on hippocampal activity. Also, a significant effect of *time* was observed ($F_{(1,12)} = 25.3357$, $P = 0.0002$), such freezing response was lower at a remote time point when compared to one day after training, suggesting that the memory had weakened over time. The lower freezing levels displayed in the 1A experiment seems to be just a local spurious effect – probably due to the immobilization procedure needed for the drug infusion – since in retest session (data not shown) where no previous immobilization was necessary, freezing response was much higher remaining similar to that observed 24 h after training. Also, animals that were directly exposed to the same footshocks (4 × 0.5 mA/1s) before immediate removal were not able to establish a contextual association, demonstrating no freezing expression (data not shown).

Since previous works demonstrated that the retrieval of remote memories is often supported by ACC (Haubrich et al., 2016; Frankland, 2004; Maviel, 2004), we chose to evaluate whether the ACC would also be able to support the acquisition of a learning event subsequent to a memory previously consolidated at the systems level. To this end, animals were infused with $GABA_A$ agonist muscimol directly into ACC before the second training. Two-factor mixed design ANOVA revealed no effect of *context* ($F_{(1,14)} = 1.3877$, $P = 0.2584$), *drug* ($F_{(1,14)} = 0.1971$, $P = 0.6638$) or *context* ∗ *drug* interaction ($F_{(1,14)} = 1.2268$, $P = 0.2867$) (Fig. 1B).

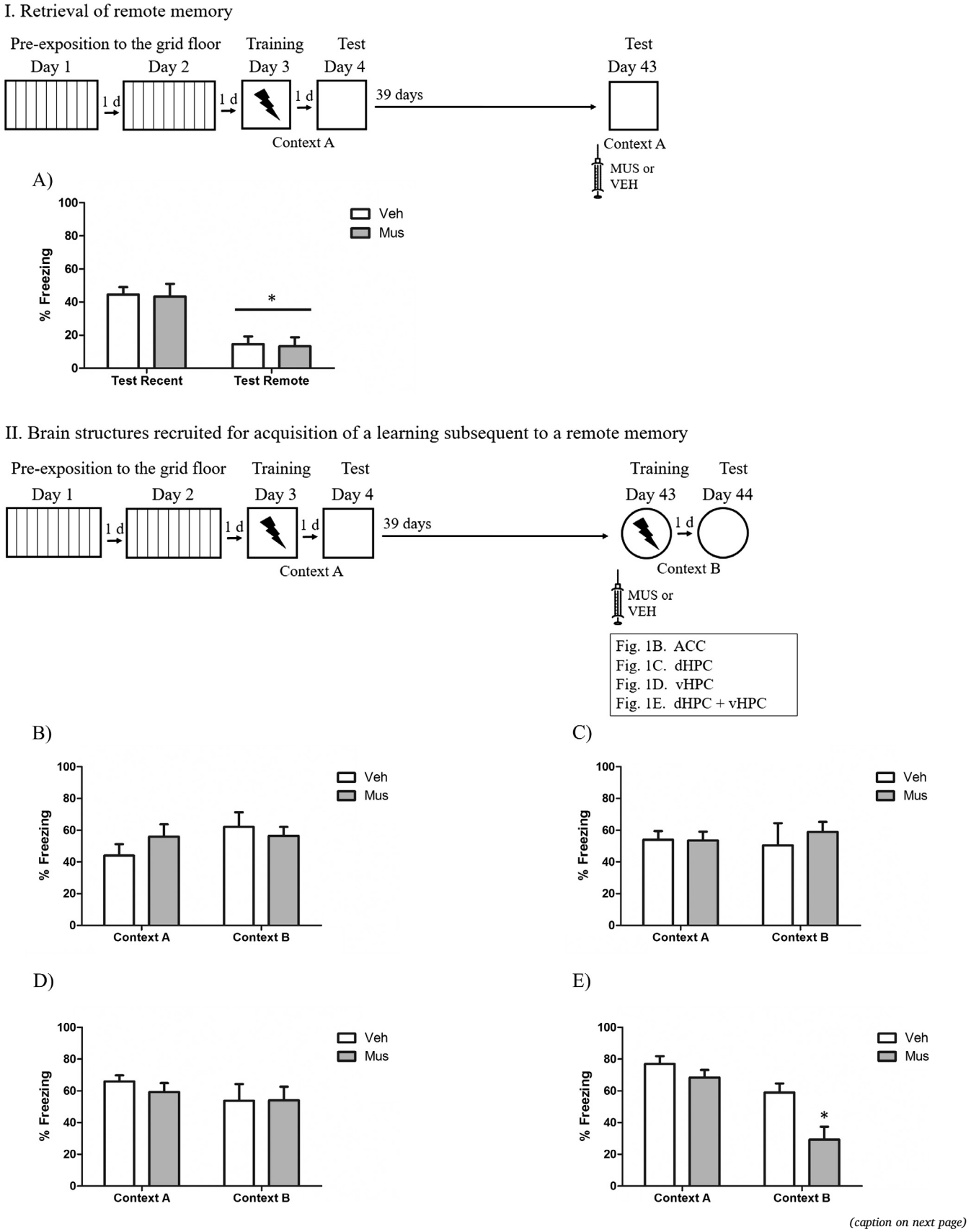
Considering that the inactivation of the ACC did not impair acquisition of subsequent learning, we next tested whether hippocampus would once again be required for subsequent contextual fear conditioning. First, dorsal (dHPC) or ventral hippocampus (vHPC) requirement were evaluated separately. After that, simultaneous inactivation of both areas (dHPC + vHPC) was performed to avoid a possible compensatory effect that could occur between both hippocampal subregions (de Hoz & Martin, 2014; Wang et al., 2012). Two-factor mixed design ANOVA revealed that dHPC inactivation did not cause memory impairment. There was no effect of *context* ($F_{(1,14)} = 0.1776$, $P = 0.6798$), *drug* ($F_{(1,14)} = 0.020$, $P = 0.8886$) or *context* ∗ *drug* interaction ($F_{(1,14)} = 0.5443$, $P = 0.4728$) (Fig. 1C) were observed. Likewise, vHPC inactivation did not cause memory impairment; Two-factor mixed design ANOVA revealed no significant effect of *context* ($F_{(1,17)} = 0.1299$, $P = 0.7229$), *drug* ($F_{(1,17)} = 2.3763$, $P = 0.1415$) or *context* ∗ *drug* interaction ($F_{(1,17)} = 0.3814$, $P = 0.5450$) (Fig. 1D). However, 2 × 2 mixed-model ANOVA revealed that inhibition of the entire hippocampus (dHPC + vHPC) caused a significant effect of *context* ∗ *drug* interaction ($F_{(1,17)} = 5.7585$, $P = 0.02814$). Newman-Keuls post-hoc analysis showed that the MUS group demonstrated a learning impairment when compared to the VEH group in context B ($P = 0.0120$) (Fig. 1E).

Therefore, our results collectively demonstrate that only the inactivation of the entire hippocampus (Fig. 1E) was able to impair the encoding of a learning event subsequent to a similar memory previously consolidated at systems level (Fig. 1A). In contrast, inactivation of just the dorsal (Fig. 1C) or the ventral (Fig. 1D) HPC, or of the ACC (Fig. 1B), did not impair memory retrieval. These results suggest that at least one hippocampal subregion is required for acquisition of an experience-dependent learning following a previously acquired, systems-consolidated memory.

3.2. Hippocampal synaptic plasticity induced by the first learning has a time-dependent influence on the experience-dependent learning

Since the hippocampus seems to be required for learning subsequent to a systems consolidated memory, we then investigated whether hippocampal plasticity induced by the first experience could still influence the subsequent learning at a remote time point. Our prediction was that the plasticity mechanisms would "reset", since the older memory is already independent of hippocampus. In the last experiment we demonstrated that at least one hippocampal subregion is reengaged for the acquisition of the subsequent learning. Thus, if the hippocampus is again required to memory encoding, it is possible that a resetting of plasticity mechanisms would also occur. For comparison, an experience-dependent learning experiment was performed at a recent (3 days) or remote (40 days) time point relative to the previously formed memory.

In both experiments animals were simultaneously infused with the NMDAR-antagonist AP5 into both the dHPC and vHPC before the second fear conditioning session. In the 3-day interval, two-factor mixed design ANOVA revealed a significant effect of *context* ∗ *drug* interaction ($F_{(1,13)} = 14.6454$, $P = 0.0021$). In this experiment, Newman-Keuls *post-hoc* analysis indicated that subsequent conditioning in context B was not reduced for NMDAR-antagonist AP5 ($P = 0.8269$), suggesting that learning subsequent to a previously consolidated recent memory remains independent from NMDARs (Fig. 2A). Additionally, AP5 impaired the learning of the first training ($P = 0.0370$), an effect that was not affected by pre-exposures to the grid floor. This result confirms the already established idea that first-time learning requires NMDAR while similar, subsequent learning does not (Bannerman et al.,



(caption on next page)

1995; Tayler et al., 2011; Wiltgen et al., 2011; Wang et al., 2012).

In contrast, at the 40-day interval, two-factor mixed design ANOVA demonstrated a significant *context* ∗ *drug* interaction ($F_{(1,14)} = 12.8449$, $P = 0.0029$), and Newman-Keuls *post-hoc* analysis showed that the AP5 group had a learning impairment when compared to the VEH group in *context B* ($P = 0.0368$), confirming that learning subsequent to a

Fig. 1. Acquisition of an experience-dependent learning after systems consolidation of a previous memory requires the hippocampus and does not recruit the ACC. (I and II) Experimental design. Rats were pre-exposed to the grid floor (day 1 and day 2) for 10 min, fear conditioned in context A (day 3) and tested in the same context (day 4). In the first experiment, animals were then re-tested on context A 40 days after training. In all the following experiments, rats were trained on context B 40 days after training on context A. Vehicle or muscimol (GABAa agonist) was infused bilaterally into target regions immediately prior to training in context B. Contextual fear memory (% freezing) was assessed 24 h after each training session. (A) Retrieval of remote memory is independent from the entire hippocampus (N = 7, 7). (B) Pharmacological inactivation of ACC using muscimol did not impair subsequent, experience-dependent learning when animals have a previously systems-consolidated, remote memory (N = 7, 9). Encoding of a learning subsequent to a memory previously consolidated at the systems-level is impaired only when the entire hippocampus ((E), N = 9, 10), but not when just its dorsal ((C), N = 7, 9) or ventral longitudinal ((D), N = 9, 10) divisions, are inactivated. Data represented as percent of freezing time during test session and expressed as mean ± S.E.M. (*) Significantly different from the respective control group ($P < 0.05$; effect of groups, two-factor mixed design ANOVA).

remote memory once again requires NMDAR in the hippocampus (Fig. 2B).

4. Discussion

Our results indicate that learning subsequent to a systems-level consolidated, remote memory (Fig. 1A) is not supported by ACC (Fig. 1B) and requires at least one functional subregion of the hippocampus (ventral or dorsal – Fig. 1C, D and E). Additionally, synaptic plasticity induced by the first learning has a time-dependent influence on the experience-dependent learning. It is NMDAR-independent when experience-dependent learning occurs at a recent time point (Fig. 2A) and becomes once again dependent on NMDAR when it occurs at a remote time point (Fig. 2B).

Contextual aversive memories are known to depend on the dorsal hippocampus which plays a critical role in processing cognitive maps (Bird & Burgess, 2008; Cohen, 2015; Moscovitch et al., 2005; Martin & Clark, 2007; Nadel, Hupbach, Gomez, & Newman-Smith, 2012). However, representation of the environment loses details over time, a phenomenon termed memory generalization, which has also been correlated with systems consolidation (Winocur, Moscovitch, & Sekeres, 2007). Hence, the hippocampus has a time-dependent role in memory retrieval that parallels the progressive take over of cortical areas (Anagnostaras et al., 1999; Frankland, 2004; Kim & Fanselow, 1992; Maviel, 2004), and the consequent increase in memory generalization (De Oliveira Alvares et al., 2013; Moscovitch, Nadel, Winocur, Gilboa, & Rosenbaum, 2006; Wang, Teixeira, Wheeler, & Frankland, 2009). In some situations, hippocampal neurons may still be involved in the recall of remote memories especially those with episodic details (Frankland & Bontempi, 2005).

Nevertheless, research on systems consolidation usually targets the dorsal division of the hippocampus precluding wider conclusions involving other subregions such as the ventral hippocampus (Anagnostaras et al., 1999; Frankland, 2004; Kim & Fanselow, 1992; Maviel, 2004; Teixeira et al., 2006). Although dHPC and vHPC have slightly different circuitries, projecting to different brain regions (Bannerman et al., 1999; Biedenkapp & Rudy, 2008; Hunsaker, Fieldsted, Rosenberg, & Kesner, 2008; Jankord et al., 2011; Padilla-Coreano et al., 2016; Phillips & LeDoux, 1992; Richmond et al., 1999; Zhang, Bast, Xu, & Feldon, 2014), there are indications that one hippocampal subregion may compensate for the other when the other happens to be inactivated (de Hoz & Martin, 2014; Keinath et al., 2014; Wang et al., 2012).

In this work, we inactivated both dHPC and vHPC to determine whether remote memory retrieval is independent of the entire hippocampus. Our data demonstrates that whole hippocampus inactivation does not impair remote memory retrieval, supporting the standard model of systems consolidation (Fig. 1A). This is in accordance with

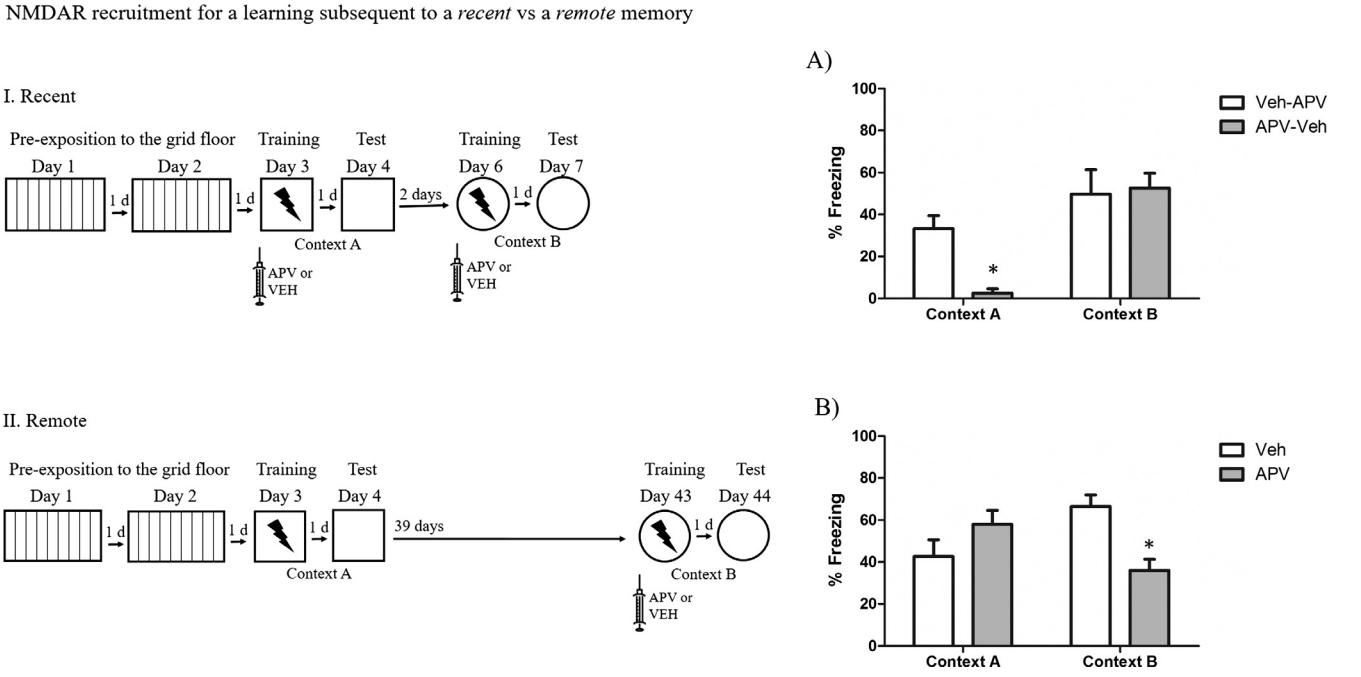


Fig. 2. Learning subsequent to a recently consolidated memory is NMDAR-independent, while learning subsequent to a remote, systems-level consolidated memory once again requires NMDAR in the hippocampus. (I and II) Experimental design. Rats were pre-exposed to the grid floor (day 1 and day 2) for 10 min, fear conditioned in context A (day 3) and tested in the same context (day 4). Animals were then trained on context B (day 6) to evaluate the influence of recent memory on subsequent learning plasticity. Vehicle or APV (NMDAR antagonist) was infused bilaterally into target regions immediately prior to training in context A and B. For remote memory, animals were trained on context B 40 days after training in context A. Vehicle or APV was infused bilaterally into target regions immediately prior to training in B. Contextual fear memory (% freezing) was assessed 24 h after each training session. (A) In a recent time point, first-time learning is impaired by NMDAR antagonist and subsequent, experience-dependent learning is not (N = 7, 8). (B) In a remote time point, subsequent learning is impaired by NMDAR-blockade (N = 9, 7). Data represented as percent of freezing time during test session and expressed as mean ± S.E.M. (*) Significantly different from the respective control group ($P < 0.05$; effect of groups, two-factor mixed design ANOVA).

Varela et al. (2016), that has elegantly demonstrated, using a chemical-genetic tool, that retrieval of a remote memory does not require hippocampal activity (Varela et al., 2016).

However, it is important to point that hippocampus disengagement for remote memory retrieval observed in our study and in the others mentioned above can be due to the slow action of the pharmacological interventions here used, which could allow for other brain areas to compensate. A faster (optogenetic) hippocampal inactivation was shown to impair a remote memory retrieval because other areas cannot compensate quickly enough for the sudden loss of the hippocampal signal (Goshen et al., 2011).

We also addressed the question of whether a cortically-established remote memory could provide support for the acquisition of a subsequent similar learning. Despite the importance of the ACC for a successful remote memory retrieval (Ding et al., 2008; Haubrich et al., 2016; Maviel, 2004; Teixeira et al., 2006), our findings demonstrate that the ACC does not play an essential role in the acquisition of a subsequent, remote learning (Fig. 1B). In accordance to our results, Tse et al. (2007, 2011) has shown that a *schema* of the prior learning persists in the cortex regardless of its inability to support subsequent learnings without the concomitant activity of the hippocampus. They also demonstrated that systems consolidation might be accelerated when the animals already possess a cortical *schema* (Tse et al., 2007, 2011). Likewise, previous studies from our laboratory have shown that systems consolidation may be accelerated by learning of novel tasks (Haubrich et al., 2016), by stress and increased shock intensity (Pedraza et al., 2016) and even by a subsequent learning experience (Pedraza, Sierra, Crestani, Quillfeldt, & de Oliveira, 2017).

Furthermore, since animals were trained twice, with a long period of time between trainings, we tried to overcome the fear generalization phenomenon by pre-exposing them to the grid floor in a different context. The grid floor was considered a relevant contextual cue since the animals received footshocks through the floor. Baseline freezing before the training sessions was measured and compared to the measurements obtained in the two successive trainings in order to evaluate fear generalization. However, grid floor pre-exposition did not reduce fear generalization (data not shown). It is also important to consider that the pre-exposure could modify subsequent learning, thus interacting with the more recent learning (Biedenkapp & Rudy, 2007; Roesler et al., 1998). To check whether this was the case, we infused AP5 into the animals previously exposed to the grid floor before the first contextual fear conditioning. Usually, NMDAR blocking is know to induce a severe impairment of memory consolidation in several behavioral tasks (Cercato et al., 2014; Fanselow & Kim, 1994; Faust, 2013; Kim et al., 1991; Morris, 1989; Young, Bohenek, & Fanselow, 1994), as well as in electrophysiological studies (Morris et al., 1986; Tsien et al., 1996). We have also observed a learning impairment when NMDAR was blocked before the first conditioning (Fig. 2A). However, in our experiment, the pre-exposure to the grid floor did not influence the plasticity mechanism originally required for first-time learning (Fig. 2A), which was still dependent on NMDAR. Other studies have demonstrated that pre-exposure to a highly similar context (Tayler et al., 2011) or to the very same context (Roesler et al., 1998) induces a learning that is independent of NMDAR. In our experiments, pre-exposure to the grid floor was performed in a different box, and thus probably not similar enough to induce a modification in the synaptic plasticity mechanisms. Thus, our findings reinforce the idea that first-time learning is dependent on NMDAR and that exposure to a context different from that used in the training session is not sufficient to induce a modification in the plasticity mechanism. Additionally, situations in which the animals have learned a different, yet also hippocampal-dependent task, such as the water maze, they are not able to engage NMDAR independency (Wiltgen et al., 2011).

In our hands, inhibition of NMDARs did not cause a reduction in the performance of the animals in the subsequent, experience-dependent learning at a recent time point (Fig. 2A). While this result differs from Wang et al., 2012, it is in agreement with studies suggesting that a prior learning alters subsequent plasticity mechanisms (Bannerman et al., 1995; Hardt, Wang, & Nader, 2009; Roesler et al., 1998; Sanders & Fanselow, 2003; Tayler et al., 2011; Wiltgen et al., 2011). Wang et al. (2012) suggested that a previous learning experience may modify how the hippocampus processes subsequent similar learning. They demonstrated the requirement of dorsal hippocampus for first-time learning while subsequent learning (in a recent time point) could be acquired and consolidated by either dorsal or ventral hippocampus. Interestingly, they have shown that subsequent learning was also impaired by a NMDAR blocker, suggesting that prior experiences does not cause a modification of the plasticity mechanisms underlying subsequent learning (Wang et al., 2012). These contrasting findings may be mainly due to differences in protocol: we have employed a lower shock intensity and a shorter interval between successive trainings, and our animals were pre-exposed twice to the grid floor. Considering that a dose-dependent effect of APV has being observed (Inglis et al., 2013), where higher concentrations cause a reduction of the subsequent learning, one could expect that a protocol with lower-shock intensity would be more sensitive to the NMDAR antagonist. Here, we have employed a lower-shock intensity (weaker learning) when compared to Wang et al. (2012), therefore it would be expected that the NMDAR antagonist would also be effective in blocking experience-dependent learning in our experimental design (Fig. 1A). However, we did not observe learning impairment. A higher number of foot-shocks (4 versus 1) could compensate the lower-shock intensity that we have used. Thus, we speculate that the metaplasticity mechanism induced by first learning possibly reduces NMDAR-dependence, while not completely removing its requirement. In this way, a dose-dependent effect is likely to be observed, where higher APV concentrations could potentially cause a reduction of subsequent learning, as demonstrated by Inglis et al. (2013). In our experiments, subsequent learning was not affected by APV when it occurs at a recent time-point after the first learning. However, memory was not impaired by the NMDAR antagonist when subsequent learning occurred at a remote time-point.

Moreover, Wiltgen et al. (2011) has suggested that the first conditioning results in a long-lasting (15 and 30 days after first training) modification of the cellular mechanisms underlying the encoding of new information. However, in that case, the NMDAR antagonist was infused systemically, preventing us from knowing which specific brain region(s) mediated the observed effects (Wiltgen et al., 2011). In the present experiments, rats were infused with an NMDAR antagonist directly into the targeted brain region, indicating that learning subsequent to a systems-level consolidated, remote memory becomes once again dependent upon NMDARs in the entire hippocampus (Fig. 2B), which suggests that the plasticity mechanisms supporting this learning were somehow “reset” in the hippocampus.

5. Conclusions

As a whole, our results have shown that (i) at least one longitudinal division of the hippocampus is necessary to encode a learning subsequent to a systems-level consolidated, remote memory, and (ii) in this region, it becomes once again dependent upon NMDARs. In our view, this suggest that the gradual decrease of hippocampal dependency, a characteristic of the systems consolidation process, may somehow involve a “reset” of the hippocampal molecular mechanisms behind the subsequent learning. By investigating the plastic changes that take place in sequential learning scenarios, we are better poised to understand the complex neurobiological nature of memory dynamics in real-life, natural conditions.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nlm.2018.02.020.

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REVIEW

Can Previous Learning Alter Future Plasticity Mechanisms?

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The dynamic processes related to mnemonic plasticity have been extensively researched in the last decades. More recently, studies have attracted attention because they show an unusual plasticity mechanism that is independent of the receptor most usually related to *first-time learning*—that is, memory acquisition—the NMDA receptor. An interesting feature of this type of learning is that a previous experience may cause modifications in the plasticity mechanism of a subsequent learning, suggesting that prior experience in a very similar task triggers a memory acquisition process that does not depend on NMDARs. The intracellular molecular cascades necessary to assist the learning process seem to depend on the activation of hippocampal CP-AMPARs. Moreover, most of these studies were performed on hippocampus-dependent tasks, even though other brain areas, such as the basolateral amygdala, also display NMDAR-independent learning.

Keywords: subsequent learning, NMDAR-independent learning, first-time learning, CP-AMPARs, reextinction

During many decades, it was thought that learning and memory were essentially a process dependent on NMDA receptors (NMDARs; LeDoux, 2000). Several studies have demonstrated how the manipulation of glutamatergic transmission interferes with memory formation. Blocking of NMDARs impairs acquisition and consolidation in different behavioral tasks, such as the water maze (Cain, Saucier, Hall, Hargreaves, & Boon, 1996; Morris, 1989; Morris, Anderson, Lynch, & Baudry, 1986), contextual fear conditioning (CFC; Fanselow, Kim, Yipp, & De Oca, 1994; J. J. Kim, DeCola, Landeira-Fernandez, & Fanselow, 1991; Young, Bohenek, & Fanselow, 1994), inhibitory avoidance (IA; Cercato et al., 2014), open field exposure (Vianna et al., 2001), object place memory (Faust, Robbiati, Huerta, & Huerta, 2013), conditioned place preference (Alaghband & Marshall, 2013), and others. Furthermore, electrophysiological studies with NMDAR antagonists (Morris et al., 1986; Tsien, Huerta, & Tonegawa, 1996) and NMDAR-knockout animals (Huerta, Sun, Wilson, & Tonegawa, 2000; Shimizu, Tang, Rampon, & Tsien, 2000; Xia et al., 2005) have also found deficits in memory formation.

However, some studies suggest these receptors would not be required for all forms of learning. A first, previous learning can alter the plasticity mechanisms of a subsequent, second learning (Bannerman, Good, Butcher, Ramsay, & Morris, 1995; Sanders & Fanselow, 2003; Roesler et al., 1998; Tayler et al., 2011; Wiltgen et al., 2010; Wiltgen, Wood, & Levy, 2011). This minireview will focus on the NMDAR-independent learning and discuss the necessary requirements for this type of learning to take place, including the underlying plasticity mechanisms.

NMDAR-Independent Learning and Hippocampus-Dependent Memory

NMDAR-independent learning was demonstrated in distinct behavioral tasks: the water maze, IA, CFC, and the radial maze (Caramanos & Shapiro, 1994). A pioneering study was performed in a water maze by Bannerman et al. (1995). In that study, the NMDAR antagonist 5-amino-phosphonovaleric acid (AP5) was able to impair the acquisition of a new memory. However, if the subjects had been previously trained in the water maze task, subsequent, second learning was not sensitive to AP5 any longer, suggesting that NMDARs were not necessary for the acquisition of the spatial representation of a new environment. Also, when the preceding learning was of spatial nature, the second, NMDAR-independent, yet still hippocampus-dependent, learning took place without restrictions; if a nonspatial water maze task is performed first, however, the NMDAR blockade deficit is prevented (Bannerman et al., 1995). In the same issue of *Nature* in which this research was presented, it was shown that the NMDAR antagonist NPC17742 (2R,4R,5S-2-amino-4,5-[1,2-cyclo hexyl]-7-phosphonoheptano acid) does not prevent spatial learning in rats that were familiar with the task requirements in a nonspatial water maze (Saucier & Cain, 1995). Therefore, involvement of hippocampal NMDARs in memory may depend on the familiarity with task requirements and the environment. Nevertheless, NMDAR antagonists might potentially cause sensorimotor disturbance and masquerade as learning defi-

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cits (Cain et al., 1996; Saucier, Hargreaves, Boon, Vanderwolf, & Cain, 1996). Recent work from Morris and colleagues have shown that sensorimotor disturbances can be avoided with a lower drug concentration, and that the pretraining phenomenon is dose-dependent (Inglis, Martin, & Morris, 2013; Morris, Steele, Bell, & Martin, 2013). All these studies display a similar finding: Under certain conditions, animals were able to learn without the NMDAR (Bannerman et al., 1995; Inglis et al., 2013; Morris et al., 2013; Saucier & Cain, 1995;).

When it comes to aversive memories—as in IA and CFC—the same phenomenon was observed. Roesler et al. (1998) showed that pretraining or preexposure to IA apparatus prevents the amnestic effect of AP5 in posterior learning. In CFC, the memory deficit caused by the infusion of an NMDA antagonist into the hippocampus was alleviated by a pretraining session in the same apparatus (Hardt, Wang, & Nader, 2009; Sanders & Fanselow, 2003). In support of these findings, Tayler et al. (2011) found that for animals trained in two different environments, learning of the second training session does not depend on the NMDAR, and the hippocampus was required for conditioning in both environments. In all of these aforementioned studies, subsequent learning remained hippocampus-dependent despite changes in learning-related synaptic plasticity.

On the other hand, some complications raised from studies included that the second training was dependent on a distinct hippocampal region (dorsal vs. ventral) or even a different brain structure (such as the entorhinal cortex; Cammarota, Bevilaqua, Köhler, Medina, & Izquierdo, 2005; de Hoz & Martin, 2014; Wang, Finnie, Hardt, & Nader, 2012). In these cases, the success of the second learning in the presence of the NMDAR antagonist may reflect an inactivity or independence from the brain area related to the first learning. For clarity, we will detail these three studies. In the first one, the work of Cammarota et al. (2005) evaluated the involvement of the striatum in IA learning by training the animals twice. The acquisition of a memory that requires the suppression of exploratory movements, such as IA, may be associated with the establishment of a habit, and habits tend to depend on the striatum (Knowlton, Mangels, & Squire, 1996). Accordingly, they found that the second IA learning involved the striatum instead of the hippocampus. Another interesting result, barely discussed, is that the acquisition/consolidation of one trial IA task required the normal functionality of type-I metabotropic glutamate receptors (mGluR1) in the striatum, despite the second round of learning being independent from mGluR1 in this brain structure (Cammarota et al., 2005). In the second study, Wang et al. (2002) showed that the first learning needs the integrity of the entire hippocampus, whereas the subsequent conditioning can be mediated either by the ventral or the dorsal hippocampus, depending on which area is needed. Additional data indicated that voltage-dependent calcium channels are not necessary for the second learning (Wang et al., 2012). The third article, from de Hoz and Martin (2014), suggests that once spatial information has been acquired in one context—for example, in the water maze—the ventral, rather than dorsal, hippocampus may mediate the consecutive learning in a distinct environment. These studies suggest that, under certain conditions, the second, subsequent learning could depend on a distinct brain area related to the first learning, and the NMDAR-independent acquisition could reflect only the independence from this particular brain area.

These findings in support of the hypothesis that learning can be acquired independently of NMDAR activation also raised the question of whether a previous learning could lead a second one, in a distinct behavioral task, to be NMDAR-independent. Thereby, a set of experiments demonstrated that animals were able to learn without NMDARs only when they were retrained on the same behavioral task. Moreover, temporal contingency remains viable after 15 or even 30 days between conditionings, and the second, subsequent learning could still take place without relying on NMDARs, suggesting a long-lasting change in the cellular mechanisms that were used to encode the related new information (Wiltgen et al., 2011).

Plasticity Mechanisms Related to NMDARs

NMDARs are ionic channels assembled from four subunits (a heterotetramer). They may be composed of GluN1, GluN2 (GluN2A-D), and GluN3 (GluN3A-B) subunits. The biophysical properties—for example, receptor permeability—and physiological roles of synaptic NMDARs are dependent on their subunit composition (Ladépêche, Dupuis, & Groc, 2014).

The NMDAR plays an essential role in the plasticity process by enabling calcium influx. Calcium acts as a second-messenger inducing activation of several intracellular biochemical cascades that lead to protein synthesis and synaptic strengthening. A central Ca^{2+}-sensitive protein that regulates these biochemical effects is calmodulin (CaM). In response to changes in intracellular Ca^{2+}, both CaMK IV and certain isoforms of CaMK II may influence gene expression. Moreover, CaMK II is directly involved in the modulation of neuronal adaptive responses regulating a large amount of substrates, such as MAP2, synapsin 1, and the GluA1/AMPA subunit. Ca^{2+} concentration influences cyclic AMP levels by activation of CaM-dependent adenylate cyclases, and also modulates the Ras signaling pathway. The activation of the Ras pathway results in the sequential activation of Raf, MEK-1, MAP kinases and ribosomal S6 kinases (Rsks), inducing gene expression (Konur & Ghosh, 2005; Ladépêche et al., 2014).

In those types of learning in which plasticity mechanisms were not mediated by NMDARs, other calcium permeable receptors must be recruited in order to trigger the intracellular molecular cascades necessary to induce protein synthesis and assist the learning process. This issue will be discussed in more detail in the following section.

A Model to Explain NMDAR-Independent Learning

Wiltgen's group (Tayler et al., 2011) proposed an interesting model to explain subsequent, NMDAR-independent learning. Being trained in a task—in that case, a context fear conditioning—activates and induces plasticity in a subset of hippocampal neurons that form the memory related to that experience. The plasticity induced by this *first learning* leads to an increase in synaptic strength and the expression of novel receptor proteins, which in turn will be able to mediate plasticity despite the presence of an NMDAR antagonist. The alteration in synaptic plasticity mechanisms promoted by the first learning enables the subsequent behavioral experience to take place in the absence of NMDAR activation. Tayler et al. suggested that NMDAR-independent learning is possible only if recently engaged neurons are reacti-

vated during subsequent learning. Therefore, contextual features seem to be an important condition to induce the reactivation of the cells involved in previous learning. Hence, the secondary context must be different enough to trigger new learning while not inducing memory generalization, and, at the same time, be sufficiently similar to the first environment in order to have a considerable amount of overlapping neurons. The work of Tayler et al. demonstrated that modifications in the degree of context similarity influence the NMDAR-dependence or NMDAR-independence of subsequent learning. A moderately similar environment is required for NMDAR-independent learning, and its context contributes to reduce memory generalization favored by a highly similar one. These experiments suggest that NMDAR-independent plasticity occurs only in previously activated cells. Thereafter, when contexts were distinct enough, NMDAR activation was once again required for learning (Tayler et al., 2011).

Assuming that NMDARs were not necessary for some types of learning, the next question was which receptor promotes this learning? A likely receptor would be calcium permeable-AMPA (CP-AMPA), which is involved with the plasticity mechanisms and is permeable to Ca^{2+}. To address this issue, Wiltgen et al. (2010) engineered mutant mice with a conditional deletion of GluR2 in the CA1 region of the hippocampus and elegantly demonstrated that GluR2-lacking CP-AMPARs were a potential candidate cellular mechanism behind NMDAR-independent learning. Consistent with this well-described phenomenon in the hippocampus, NMDAR-independent learning was also observed in the barrel cortex. Experiments performed by Clem, Celikel, and Barth (2008) found that synaptic strengthening induced by a single whisker stimulation protocol initially requires NMDARs. However, ongoing whisker stimulation induces an NMDAR-independent synaptic strength, requiring mGluR and CP-AMPAR (Clem & Barth, 2006; Clem et al., 2008). In this way, these studies support the hypothesis that subsequent learning reactivated a subset of neurons related to previous learning, and that some synaptic plasticity mechanism is modified by a previous behavioral experience.

When it comes to cellular signaling pathways, calcium influx from different types of calcium-permeable receptors does not necessarily act through the same molecular cascades. For instance, when comparing the NMDAR with the CP-AMPAR, the former classically activates the Ca/CaM-dependent kinase II (CaMK II) pathway, whereas the latter requires phosphatidylinositol-3-kinase (PI3K)/MAP kinases, but not CaMK II. Hence, both CP-AMPARs and NMDARs have been shown to activate the ERK/MAPK signaling cascade and phosphorylation of cAMP response element-binding protein (CREB) through PI3K. Because CP-AMPAR-dependent plasticity rules out the activity of CaMK II, alternate factors such as Ras and PI3K may be better candidates to activate the ERK/MAPK pathway in response to calcium (Asrar, Zhou, Ren, & Jia, 2009; Perkinton, Sihra, & Williams, 1999).

An intriguing question still remains unanswered regarding the immediate early genes (IEGs) that are activated in response to calcium influx from distinct receptors. The expression of Arc and *c-fos*, for instance, is mediated by NMDAR activation, even though this receptor is not required for some learning situations. The implication in this case, is that Arc and *c-fos* expression would not be required for NMDAR-independent learning (Wiltgen et al., 2011). More studies are necessary in order to understand how different calcium receptors mediate the activation of IEG.

NMDARs-Independent Learning and Extinction Memories in the Basolateral Amygdala (BLA)

Classically, activation of NMDAR in the BLA is required for context fear conditioning. In contrast to the idea that plasticity mechanisms in the hippocampus are dynamic and can be altered by prior behavioral experience, some studies reported that this phenomenon does not occur the same way in the BLA. Laurent and Westbrook (2009) demonstrated that neither context preexposition (familiarity) nor training twice in an identical context is enough to induce NMDAR-independent learning in the BLA. Moreover, subsequent fear conditioning to different stimuli, for example, first to light and second to tone, or performed in distinct contexts, remained dependent upon NMDAR activation (Lee & Kim, 1998). reset>However, another work suggested that the plasticity mechanisms are changed because of repeated training, being initially dependent, but not later, on the NMDA (Pistell & Falls, 2008). In addition to the contrasting results, there is a lack of research evaluating the dynamic of other types of glutamate receptors (e.g., CP-AMPARs and mGluRs) after retraining, or subsequent training in a similar context. When we manage to understand glutamate receptor dynamics in the BLA induced by subsequent learning, we would be able to tackle the particularities of local NMDA-independent learning mechanisms.

On the other hand, thinking more broadly about mnemonic processes, prior learning might influence not only a similar experience but also subsequent learning with different, even opposite meaning, as is the case for an extinction memory. Nowadays, it is widely accepted that an extinction memory is a second, new memory trace, which has a *transient* inhibitory effect upon a previously consolidated memory. For aversive memories, for which a conditioned stimulus (conditional stimulus [CS]) is paired with an unconditioned stimulus (US), an extinction memory is formed after a long or repeated reexposition to the CS without the presence of the US, leading to a decline in the expression of the conditioned response (Berman & Dudai, 2001). Many studies have extensively characterized extinction memories, and their behavioral and molecular bases are now at least partially well established. Regarding the importance of NMDARs during extinction process, animals cannot learn to inhibit fear related to the original conditioning if NMDARs are blocked (Quirk & Mueller, 2008; Zimmerman & Maren, 2010). Furthermore, it has been demonstrated that CP-AMPARs are crucial to the erasure of the memory trace in a specific protocol of extinction, as well as in the memory reconsolidation process in the lateral amygdala (Clem & Huganir, 2010; Hong et al., 2013).

Additionally, there is an indication that learning of extinction is also modulated by previous experience. For instance, Langton and Richardson (2008, 2009) demonstrated that when D-cycloserine (a NMDAR partial agonist) is used to facilitate, and MK-801 (a NMDAR antagonist) to prevent, the first extinction, the second extinction becomes insensitive to either NMDAR agonists or antagonists, provided that both extinctions involve the same CS. Conversely, Chan and McNally (2009) showed that CS familiarity influences the sensitivity to the NMDAR antagonist. Thereby, these data suggest that reextinction is also an NMDA-independent process, in a nice analogy to what we have been observing in first-second learning situations.

In agreement with reports that demonstrated that subsequent learning could depend on distinct brain areas related to the first learning, studies with pharmacological inactivation have, however, indicated that the BLA may not be required for memory reextinction (J. H. Kim & Richardson, 2008; Laurent, Marchand, & Westbrook, 2008). On the other side, the ventral medial prefrontal cortex has been shown to play an important role in the effectiveness of extinction and reextinction processes (Morgan, Schulkin, & LeDoux, 2003).

All these extinction-related findings support the idea that a previous learning experience—either the acquisition or the inhibition of fear—can alter the molecular mechanisms that trigger, respectively, the acquisition or the extinction of subsequent memories not only in the hippocampus but also in the amygdala.

Concluding Remarks

Currently, despite having accumulated a relatively elaborate understanding about memory plasticity behind the different phases of memory processing, we lack information on quite ordinary situations such as how a (first-time) learning may affect a second, subsequent one. Here, we reviewed some studies evaluating specific conditions and plasticity substrates (receptors, etc.) that seem to be involved in what is being called a "subsequent learning" process. The evidence converges, in that previous learning can modify the plasticity mechanism of subsequent learning, frequently turning it into an NMDAR-independent process. It is necessary to further investigate these events in order to better understand (a) the intracellular molecular cascades necessary to support these otherwise different learning mechanisms, (b) the role of temporal contingency between sequential learnings, and (c) which brain areas may be recruited by each task. The fact that animals are not "blank slates," but instead are ever learning, just reinforces the importance of knowing these and other specific conditions able to induce this switching between plasticity states that is not only extremely relevant but also common.

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